University of Alberta

Studies on the signal transduction pathway of an osmotic stress inducible promoter

by

Phyllis Louise Dale

A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Doctor of Philosophy

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The undersigned certify that they have read, and recommended to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled "Studies on the signal transduction pathway of an osmotic stress inducible promoter" submitted by Phyllis Louise Dale in partial fulfilment of the requirements for the degree of Doctor of Philosophy in Plant Science.

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Abstract

An ABA- and drought-inducible promoter from *Craterostigma plantagineum*, fused to bacterial luciferase (CDeT27-45*luxF*) was introduced into *Nicotiana BY-2* protoplasts by Ca-Peg transfection. The CDeT27-45 promoter was weakly expressed and up-regulated 2-fold by exogenous ABA application. Increased luciferase activity with 5'-truncations of the CDeT27-45 promoter up to -335 bp was observed.

CdeT27-45*luxF* and its 5'-truncated derivatives were introduced into *Nicotiana tabacum* by *Agrobacterium*-mediated transformation. Analysis of luciferase activity generated by these constructs revealed that the CDeT27-45 promoter was highly expressed in mature seed and induced by hyperosmotic stress in leaf tissue. The CDeT27-45 promoter was not inducible in tobacco by exogenous ABA application or wilting.

Suspension cell cultures were generated from CDeT27-45*luxF* transformed leaf tissue in order to investigate the osmotic stress signal transduction mechanism. Luciferase activity was not induced by tobacco cell wall fragments present in conditioned medium or by added pectin, but osmotic induction was suppressed by treatment of suspension cultures with conditioned medium or endopolygalacturonase digested pectin.

Treatment of suspension cell cultures with 10 mM short-

chain fatty acids (SCFA) such as butyrate also resulted in induction of luciferase activity, particularly in medium with pH adjusted to 7.2. This finding suggested that the induction was not due to proton flux across the plasma membrane, but may have been due to increased membrane fluidization due to the high concentration of SCFA. Butyrate induction was not suppressed by pectin fragments in either conditioned medium or pectin hydrolysate. Incubation of the suspension cultures with 1% BSA was found to significantly reduce both osmotic and butyrate induction of luciferase activity.

Treatment of the suspension cultures with proteolytic enzymes such as trypsin reduced or eliminated both osmotic and butyrate induction of luciferase activity. These results suggest that an osmotic stress receptor resides in the plasma membrane where it is activated by membrane perturbation. A portion of the receptor is exposed to proteolytic cleavage which results in the loss of osmotic stress- or butyrate induction of luciferase activity.

Treatment of suspension cell cultures with cytoskeleton-disrupting compounds such as cytochalasin D and acrylamide reduced osmotic or butyrate induction of luciferase activity. This finding suggests that the cytoskeleton is involved in osmotic stress signal transduction.

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List of Abbreviations

^{32}P	phosphorous 32
ABA	abscisic acid
Amp	ampicillin
Amp^R	ampicillin resistant
ATP	adenosine 5'-triphosphate
Ax	absorbance at x nm wavelength

B(L)/B(R) left-and right-border sequences of vector T-DNAs.

BME β-mercaptoethanol

bp base pairs

BSA bovine serum albumin

Cb Carbenicillin

ccc covalently closed circular

CD cytochalasin D

cDNA complementary deoxyribonucleic acid

CI chloroform-isoamyl alcohol (24:1 volume:volume)

cpm counts per minute

CTAB cetyl trimethyl ammonium bromide

CW cell wall

d day(s)

dap days after pollination

dATP deoxyadenosine 5'-triphosphate

dCTP deoxycytidine 5'-triphosphate

Δ(lacZ)M15 expresses a fragment that complements the lac α-fragment encoded by many vectors.
Δ(lacZ)U169 the entire *lac* operon is deleted from the chromosome.

ddH2O deionized distilled water

dGTP deoxyguanosine 5'-triphosphate

DMSO dimethyl sulfoxide

dNTPs deoxyribonucleoside 5'-triphosphates

ds DNA double stranded DNA

DSP drought stress protein

DTT dithiothreitol

dTTP deoxythymidine 5'-triphosphate

ECM extracellular matrix

EDTA	ethylenediaminetetraacetic acid
endA	activity of nonspecific endonuclease I abolished
EtBr	ethidium bromide
EtOH	ethanol
F	a low copy number self-transmissible plasmid
FCCP	carbonylcyanide p-trifluoromethoxyphenylhydrazone
Fn	fibronectin
Fus	fusicoccin
g	gram(s)
GUS	β-glucuronidase
h	hour(s)
HMC	high mobility complexes
HRGP	hydroxyproline-rich glycoprotein
hsdr	mutations which abolish DNA restriction but not methylation.
hsdm	mutations which abolish both DNA methylation and restriction.
IFs	intermediate filaments
kan	kanamycin
kan^R	kanamycin resistant
kbp	kilobase pairs
kD	kiloDaltons
lacY	lactose utilization: galactosidase permease.
lea	late embryogenesis abundant proteins
leu	mutants require the amino acid leucine for growth in minimal medium.
LB	Luria broth
LMC	low mobility complexes
Lux	luciferase

M	molar = mole per liter
MFs	microfilaments
min	minute(s)
ml	milliliter(s) = 10^{-3} liter
mg	milligram(s) = 10^{-3} gram
mm	millimeter(s) = 10^{-3} meter
mM	millimolar = 10^{-3} mole
mole	6.022×10^{23} molecules
MTs	microtubules
N	normal (solution)
NOS	nopaline synthase promoter
NPT-II	noemycin phosphotransferase gene of transposon Tn5.
O/N	overnight
ODx	optical density at x nm
Ori T	origin of conjugal plasmid transfer.
Ori V	conditional origin of replication of broad-host-range plasmid RK2.
OSM	osmoticum
pA ocs	polyadenylation signal sequence derived from octopine synthase gene.
PCI	Tris equilibrated phenol-CI (25:24:1)
PD	plasmodesmata
PM	plasma membrane
P nos	nopaline synthase gene promoter.
Pro	mutants require the amino acid proline for growth in minimal media.
R	antibiotic resistance
RecA	homologous recombination abolished

relA	relaxed phenotype: permits RNA synthesis in the absence of protein synthesis.
Rif	Rifampicin
RNA	ribonucleic acid
rpm	revolutions per minute
RT	room temperature
SCFAs	short chain fatty acids
sdH_2O	sterilized distilled water
SDS	sodium dodecylsulphate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
s	second(s)
Sm	streptomycin
SSC	sodium chloride, sodium citrate
SupE	strains carry an amber-suppressor tRNA: required for the growth of some phage vectors.
TCA	trichloroacetic acid
TE	Tris-EDTA buffer
TEMED	N,N,N',N',-tetramethylethylenediamine
TGE	transient gene expression
thi-1	mutants require vitamin B1 for growth in minimal media.
Tris	Tris(hydroxymethyl)amino-methane
U	unit(s) of enzyme
UV	ultraviolet
V	volts
Vin	vinblastine
Vn	vitronectin
°C	degree Celsius
µg	microgram(s) = 10^{-6} gram

µl	microliter(s) = 10^{-6} liter
µM	micromolar = 10^{-6} moles per liter

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1 Introduction

Crop plants are often subjected to adverse environmental conditions such as drought, flooding, extreme temperatures, excessive salts and heavy metals. Agricultural losses resulting from environmental stress are significant (Le Rudulier *et al* 1984). Boyer (1982) has determined that over 40% of total insurance indemnities for crop losses in the United States from 1939 to 1978 involved drought. Consequently, the mechanisms by which some plant species can tolerate desiccation are of interest because the possible transfer of drought-tolerance mechanism(s) to agronomically important species could be beneficial to crop production. Desiccation-tolerant plants also provide an environmentally responsive system in which to investigate plant development (Blum 1988).

1.1 Dehydration tolerance of resurrection plants

The resurrection plant *Craterostigma plantagineum* Hochst. (fam Scrophulariaceae) is a South African weed which can withstand a 99% water loss and yet remain viable after rehydration (Gaff 1971). In the majority of higher plants, tolerance to protoplasmic dehydration is restricted to the seed and it is during embryogenesis

that the developing embryo acquires desiccation tolerance (Bewley *et al* 1989). Germination interrupts this state of tolerance and the emerging seedling rapidly loses the ability to survive desiccation. A unique feature of the poikilohydric resurrection plants is that they possess mature foliage which can develop the capacity to exist in a dry but viable state for long periods of time. After rehydration, dried leaves and other plant organs assume physiological activity within several hours (Gaff 1977).

Bartels *et al* (1990) have presumed that the proteins which are produced as *Craterostigma* undergoes desiccation are involved in the drought-survival mechanism of this species and have cloned from *Craterostigma plantagineum*, five desiccation-specific genes which code for specific drought stress proteins (DSP). These genes are activated in response to dehydration or application of the phytohormone abscisic acid (ABA) (Piatkowski *et al* 1990). CDeT27-45 was one of the drought-induced cDNAs which was selected for further molecular analysis. CDeT27-45 was of particular interest because it was found to hybridize to mRNA specifically induced during dehydration in *Craterostigma* leaves (Piatkowski *et al* 1990).

1.2 Characterization of CDeT27-45 protein.

The CDeT27-45 protein is 80% and 81% homologous to cotton (Galau *et al* 1993, Bartels *et al* 1993) and soybean Lea 14 protein, respectively (*BLAST* search of GenBank sequence data). Unlike C27-04 (DSP14) and C6-19 (DSP16), two proteins also synthesized in *Craterostigma* in response to desiccation, C27-45 (DSP15) protein does not belong to the dehydrins or D-11 family (Dure 1993) as it does not contain the 14-mer amino acid sequence KKGIMEKIKEKLPG, which is characteristic of members of the D-11 family. The C27-45 protein, which is 151 amino acids in length, is glycine and lysine rich which may result in increased random coil in the protein and is a characteristic of dehydrin and Lea proteins (Close 1993). It has been suggested that random coil allows proteins to preserve membrane structures by acting as surface-solvation moieties in place of water during cellular dehydration (Dure 1993). Cotton and soybean *Lea14* and C27-45 proteins define an additional family of drought-stress related proteins, the Group 4 *Lea* proteins which are slightly hydropathic throughout (Galau *et al* 1993). Therefore, although the exact function of the CDeT27-45 protein is unknown, it seems likely that this protein is involved in the protection of cytoplasmic components during desiccation.

I am interested in the mechanisms by which a plant senses a specific environmental stress and then activates a specific set of genes in response to that stress. As a model system I chose to study the signal transduction mechanism which leads to the activation of the CDeT 27-45 promoter in response to dehydration stress. Understanding the signal transduction mechanism by which the CDeT27-45 promoter was induced would extend our understanding of how drought-adapted plants are able to perceive adverse conditions such as dehydration.

This study is divided into three sections:

1 CDet27-45-luxF transient gene expression (TGE) assays in tobacco protoplasts.

2 Analysis of CDeT27-45-luxF in stably transformed tobacco plants.

3 CDeT27-45-*luxF* induction in suspension cell culture derived from transformed tobacco leaves.

In this thesis I will present an introduction to plant perception and responses to drought stress (Chapter 1), CDeT27-45 promoter deletion analysis in a heterologous host via TGE assay (Chapter 2) and whole transgenic plants (Chapter 3). Chapter 4 and 5 deal with the mechanisms which contribute to the induction of the CDeT27-45 promoter in transformed suspension culture

cells. Finally, in the conclusion (Chapter 6) I will present a model of osmotic-stress signal transduction mechanisms.

1.3 Role of osmotic adjustment by plant cells as a metabolic response to drought-stress

Drought, low temperature, and high salinity are all environmental stresses which alter cellular water balance and significantly limit plant growth and plant yield (Morgan 1984, Mundy and Chua 1988, McCue and Hanson 1990). Some physiological processes are altered in response to conditions which reduce cellular water potential, including photosynthesis, stomatal opening, leaf, stem and root growth (Davies and Zhang 1991, Hanson and Hitz 1982, Rhodes 1987). As well as physiological changes which act to reduce the rate of water loss, a number of metabolic changes occur (Rhodes *et al* 1986). For example, some plant species synthesize and accumulate a number of low molecular weight, osmotically active compounds such as sugar alcohols (Turner 1979), amino acids (Morgan 1984, Good and Zaplachinski 1994) and organic acids (Voetberg and Sharp 1991). Proline is one of the most widely distributed osmolytes accumulated under stress conditions (Machackova *et al* 1989, Delauney and Verma 1993). Although their exact function is

unknown, accumulation of these compounds may lead to osmotic adjustment via an increase in the intracellular osmotic potential of the cell (Yancy *et al* 1982, Handa *et al* 1983, Rhodes 1987).

1.4 Abscisic acid as well as osmotic-stress induces specific gene expression.

The expression of several late embryogenesis-abundant genes such as D19h in *Arabidopsis* and *Em* in wheat as well as the *rab* and dehydrin genes of rice and barley are induced by ABA (Skiver and Mundy 1990, Bostock and Quatrano 1992). The protein products of these genes are thought to act as osmoprotectants of cellular proteins and structures or to maintain adequate hydration levels through their water-sequestering characteristics (Dure *et al* 1989, Dure 1993, Close *et al* 1993, Bray 1993).

The correlation between environmental stress and increases in ABA level has been well established (Hsiao 1973, King 1976, Cohen and Bray 1990, Chandler and Robertson 1994). ABA is synthesized through the carotenoid biosynthetic pathway (Parry 1993). The cleavage of 9'-cis-neoxanthin results in the intermediate to ABA, xanthoxin, which is oxidized to ABA-aldehyde, then converted to ABA by ABA-aldehyde oxidase (Parry

1993). Transcription and translation are required for ABA biosynthesis during stress (Guerrero and Mullet 1986), indicating that ABA biosynthetic enzymes or other proteins in the pathway must be synthesized for elevated levels of ABA to accumulate and before ABA-requiring genes can be induced. Therefore induction requires a period of time between onset of dehydration stress and accumulation of sufficient levels of ABA. For example, it has also been shown by Wilen *et al* (1990) that seed storage protein mRNA accumulates rapidly in *Brassica napus* microspore embryos in response to ABA application or more slowly when cultured on high osmoticum. They found that an increase in endogenous ABA occurs in response to high osmoticum and precedes seed storage protein message accumulation.

ABA-deficient mutants have provided genetic evidence for the independent, but overlapping roles of ABA and osmotica in the stress induced acclimation process (Reid 1990, Pla *et al* 1991, Finkelstein 1993). *Arabidopsis thaliana* has been shown by Nordin *et al* (1991) to synthesize *lti140* mRNA in response to exogenous ABA application or in response to cold- or desiccation-stress, whereas *A thaliana* ABA mutants, such as *abi-1* (ABA-insensitive), synthesize *lti140* mRNA only in response to cold- or desiccation- stress. Similarly,

Yamaguchi-Shinozaki and Shinozaki (1993a) found that the *A thaliana* rd29 gene, which is induced by desiccation, was also induced by desiccation in *A thaliana* abi-1 and aba-1 (ABA synthesis) mutants. Using *Arabidopsis* abi-1 and aba-1 double mutants, Ooms *et al* (1994) found that the desiccation-tolerance could be restored in the *Arabidopsis* double mutant seeds which, although viable, are desiccation-intolerant. In these mutant seeds, desiccation-tolerance was shown to be restored by either slow drying, treatment with osmotica or, due to the leakiness of the abi-1 mutation, treatment with ABA. Further evidence in support of the concept of at least two separate drought-responsive pathways has been reported by Espelund *et al* (1995). They found that barley embryo-specific mRNA B15C, as well as *rab* and dehydrin mRNAs, which are induced in embryo as well as vegetative tissue, were expressed in response to mannitol in immature barley embryos in the presence of norflurazon which decreased the endogenous embryo ABA levels significantly. These findings suggest the existence of at least two separate signal transduction pathways responding to desiccation: an ABA-responsive and an ABA-independent pathway (Bray 1993, Nordin *et al* 1993, Ooms *et al* 1994).

1.5 Specific genes are induced in response to osmotic stress.

Changes in gene expression in response to osmotic stress as well as during developmentally controlled dehydration during seed maturation (Kermode and Bewley 1987), have been documented in rice (Claes *et al* 1990) and barley (Bartels *et al* 1988). *Pisum sativum* genes 15a and 7a, which encode for a thiol protease and a MIP (major intrinsic protein of bovine lens fibre gap junctions)-related protein, respectively, are induced by desiccation and not by ABA in leaf tissue (Guerrero *et al* 1990) as are similar *A thaliana* proteins rd19 and rd21 (Yamaguchi-Shinozaki *et al* 1992). *Trg*-31 gene, which has sequence similarity to bovine and *E coli* putative membrane transport proteins, has been shown to be transcribed within 30 minutes of loss of leaf turgor (Guerrero and Crossland 1993). These results indicate that there are genes induced in response to desiccation which do not require ABA as a component of their desiccation-stress signal transduction pathway. Although drought, low temperature and salinity all apply osmotic stress, the pattern of genes induced in response to these stresses is not identical. This suggests that the signals from drought, temperature and salt stress and ABA may act at different points upon an interconnected network of events

linking osmosensing to adaptive response.

1.6 Signal transduction mechanisms by which plant cells perceive osmotic stress.

Plants must have a variety of perception mechanisms that allow the sensing of environmental information and evoke appropriate physiological and developmental responses. The environmental information must first be perceived, requiring the existence of specific receptor/sensing systems, then the information must be transduced into a cellular signal which initiates the required physiological change.

It is apparent from the literature that many avenues of signal transduction exist which may link the perception of environmental stress to changes in gene expression. Decreases in turgor and osmotic potential (Bray 1993, Vierling and Kimpel 1992), rapid changes in leaf translatable RNA (Guerro and Mullet 1988), ABA accumulation (Lång and Palva 1992, Chandler and Robertson 1994), change in jasmonate concentration (Farmer *et al* 1992, Maslenkova *et al* 1992), redistribution of intracellular Ca^{2+} (Trewavas and Knight 1994) and changes in specific phosphoinositide levels (Chen *et al* 1991, Drøbak BK 1992) have been documented to occur in response

to particular environmental stresses. Such fluctuations, either singly or in combination, may be involved in linking perception of environmental stress to changes in gene expression.

1.7 The importance of the cell wall in plant development and perception of environmental stress.

Plant cell walls are important determinants in cell differentiation. For example, cell wall fragments binding to thin-layer tobacco explants have been shown to regulate further cell differentiation and morphogenesis (Eberhard *et al* 1989). The cell wall is also necessary to fix axis polarity in *Fucus* embryos in response to light (Kropf *et al* 1989) and to determine *Fucus* embryo morphogenesis (Berger *et al* 1994).

The attachment of cell walls to the plasma membrane at focal adhesion sites has been documented microscopically (Oparka 1994a). Isolation of plant cell wall proteins which might be involved in linking the cell wall to the plasma membrane has resulted in the characterization of protein families which have also been shown to be involved in plasma membrane-extracellular matrix interactions in animal cells (Hitt and Luna 1994, Lloyd 1989). Plant cell wall proteins have been divided into 5

major groups: hydroxyproline-rich glycoproteins (HRGPs), glycine rich-proteins (GRPs), proline-rich proteins (PRPs), the solanaceous lectins, and the arabinogalactan proteins (AGPs) (Showalter 1993).

HRGPs form long relatively rigid rods with an α-helical core of protein wrapped round with arabinogalactan oligomers (Cassab and Varner 1988). HRGP -antigen has been found to be present on protoplast plasma membrane but not if cells were plasmolysed first in hyperosmotic medium. In plasmolysed cells, the plasma membrane has pulled away from the cell wall due to water-loss from the cytoplasm and concomitant reduction in cell volume. However, if protoplasts were generated by treatment with cell wall-digesting enzymes, HRGP-antigen could still bind to its cognate protein on the plasma membrane (Pont-Lezica *et al* 1993). This finding indicates that HRGP is firmly attached to the cell wall as well as linked to the plasma membrane. HRGPs such as extensins have been proposed to be structural proteins that may also function in development and plant defense because the positively charged extensin molecules interact ionically with negatively charged surfaces of certain plant pathogens elicitors (Showalter 1993). Qi *et al* (1995) have recently presented data from solubilization studies using cotton suspension cell walls, that cotton extensin forms pectin-

protein cross links in addition to protein-protein cross-links.

Another group of plasma-membrane associated HRGPs is arabinogalactan proteins (AGPs) which protrude from the membrane into the cell wall (Pennell *et al* 1989, 1991, Roberts 1990, Wyatt and Carpita 1993). These may also function as cell surface extracellular-matrix-binding protein since they particularly bind to pectic fractions of the wall (Baldwin *et al* 1993). Carbohydrate moieties similar to those present in AGPs, the arabinogalactans, also exist in the cell wall unattached to protein (Showalter 1993) and are also released into the medium by rose cell suspension cultures (Komalavilas *et al* 1991). AGPs are characterized by high levels of galactose and arabinose and abundant quantities of alanine, serine and hydroxyproline. Herman and Lamb (1992) have shown that AGPs are localized to the plasma membrane in tobacco cells.

In mammalian cells, attachment of the plasma membrane to the extracellular matrix (ECM) is mediated by a class of plasma membrane receptors termed integrins (Ruoslathi and Pierschbacher 1987) and a family of ECM-localized adhesive glycoproteins such as fibronectin and vitronectin (Hynes 1992). A number of integrins can

recognize the amino acid sequence Arg-Gly-Asp (RGD) which has been shown to exist as single or multiple unit(s) in polypeptide chains of attachment glycoproteins such as vitronectin and fibronectin (Ruoslahti and Pierschbacher 1987). Synthetic peptides containing the RGD sequence can competitively inhibit the interaction between integrins and RGD containing glycoproteins isolated from the ECM (Ruoslahti and Pierschbacher 1987). Schindler et al (1989) showed that treatment of soybean suspension culture with synthetic RGD-containing peptide resulted in aberrant cell wall-plasma membrane connections. Wayne *et al* (1992) found that treatment of *Chara* cells with RGDS inhibited gravisensing in a concentration-dependent manner while RFDS (F = phenylalanine substituted for glycine) had no effect. In animal cells, the cytoplasmic domain of integrin interacts with actin cytoskeleton or it may bind to intermediate filaments (Quaranta and Jones 1991) which potentially involves the cytoskeleton in signal transduction (Ingber 1991). For example, the β-casein gene in mouse mammary cells has been shown to be transcriptionally regulated through extracellular matrix binding (Schmidhauser *et al* 1990, Streuli *et al* 1991).

1.8 Signalling mechanisms which involve glycoprotein binding to receptors on the plasma membrane.

Glycopeptides released by plant pathogens have been shown to be potent signal molecules or elicitors which induce defense or stress responses in the host plant (Dixon and Lamb 1990, Hahlbrock and Scheel 1989, Parker *et al* 1991). Cheong and Hahn (1991) have shown that the plasma membrane of soybean contains the binding sites for a glucan elicitor derived from the fungal pathogen *Phytophthora megasperma* f sp *glycinea*. However, some virulent fungi have mechanisms to prevent plant stress responses. For example, the induction of phytoalexin in soybean in response to glucan elicitor produced from *Phytophthora* cell walls is suppressed by invertase produced by a pathogenic race of *Phytophthora*. This suppressor activity appeared to be due to the carbohydrate moiety of invertase, because it was abolished by periodate oxidation which oxidizes carbohydrates, but not by heat treatment which would denature proteins (Zeigler and Pontzen 1982). Basse and Boller (1992) presented evidence for the existence of suppressors, derived from elicitor-active glycopeptides, which competitively inhibited elicitor binding. They showed that the oligosaccharides released upon glucosaminadase-H treatment of elicitor glycoprotein derived from yeast extract, completely inhibited the elicitor activity of the yeast glycopeptides on tomato leaf suspension cells. This suppressor activity was

abolished by treatments which specifically affect carbohydrate moieties, such as periodate oxidation and mannosidase digesion. They suggested that these suppressors bind to one of the elicitor recognition sites without producing a signal and thereby prevent the induction of the stress responses by the corresponding elicitor.

1.9 Signal transduction via polarization changes in plant cell membranes and/or fluxes in cytoplasmic pH.

Tobacco leaf cells have a markedly negative membrane potential while plasmolysed cells i.e. those cells whose plasma membrane has shrunk away from the cell wall due to water-loss from the cytoplasm and concomitant reduction in cell volume, or protoplasts which have been generated with cell wall-digesting enzymes, acquire a positive membrane potential (Li and Delrot 1987, Racusen *et al* 1977). Racusen *et al* (1977) demonstrated that turgor pressure affected membrane potential and the extent of cell wall regeneration of recovering protoplasts was important for restoration of a negative membrane potential.

Drought stress is known to cause decreases in turgor

pressure, cell volume and osmotic potential, which could singly or in combination, act as desiccation signals (Oparka 1994b). Similarly, cells incur water loss when a hypertonic, non-permeating solute such as mannitol is applied as an external solution. The result is decreased protoplast volume, decreased cell turgor and separation of the the plasma membrane from the cell wall (ie plasmolysis) (Stadelmann and Stadelmann 1989, Oparka 1994b). In addition, there is a change in membrane potential and in membrane resistance (Reid and Overall 1992). Addition of osmoticum to tobacco leaf cells has been shown to cause a change in membrane potential and cytoplasmic pH due to proton efflux (Reinhold *et al* 1984, Racusen *et al* 1977). The lower the turgor pressure, the greater the extent to which the membrane becomes depolarized (Li and Delrot 1987).

1.10 The osmotic stress signal transduction pathway may be mechano-sensitive and involve cytoskeletal components.

Hypertonic solutions have long been known to produce rapid plasmolysis, that is the shrinking of the cell bounded by the plasma membrane within the rigid cell wall, as water moves out of the cell to the lower water potential of the surrounding medium (Oparka 1994b). However, points of attachment or focal adhesions remain

between the plasma membrane and the cell wall. This observation, as well as the presence of glycoprotein epitopes which are similar to ECM-plasma membrane adhesion molecules in animal cells, supports the model of an interlinked cytoskeleton/plasma membrane/cell wall in plant cells (Wyatt and Carpita 1993).

The plant cell cytoskeleton has been demonstrated to contain at least two filamentous networks that are structurally equivalent to microfilaments (MFs) and microtubules (MTs) (Lloyd 1989, Traas *et al* 1987). Evidence for plant cell intermediate filaments (IFs) based on IF-antibody binding has also been presented (Goodbody *et al* 1989, Hargreaves *et al* 1989), although the existence in plant cells of epitopes similar to those of animal cell IFs does not necessarily mean equivalent proteins are present (RJ Cyr, Pennsylvania State University, pers comm). The filamentous networks in plants may be further subdivided into at least four cellular domains: nuclear, transvacuolar, subcortical cytoplasm, and plasma membrane associated (Traas *et al* 1987). Functionally, the plant cytoskeleton appears to be composed of separate domains. The nuclear and transvacuolar domains may be involved in cell division and nuclear position (Lloyd 1989, Traas et al 1987, Katsuta and Shibaoka 1988). The subcortical domain may be

involved in cytoplasmic streaming (Traas 1990), while the plasma membrane associated network may be important for secretion, endocytosis, cell wall biosynthesis and initiating transmembrane signals (Traas 1990). It appears that some of the networks are interconnected within a dynamic cytoskeletal grid composed of microfilaments and microtubules (Lloyd 1989, Traas *et al* 1987, Traas 1990). Microscopic evidence has shown that the nucleus sits within an F-actin cage which is continuous with transcytoplasmic actin cables that appear to anchor the nucleus to the plasma membrane (Traas *et al* 1987). Treatment of cells with cytochalasin D which causes dissociation of actin filaments (Abe and Takeda 1989) allowed nuclear displacement to occur (Katsuta and Shibaoka 1988) and also causes significant decrease in actin filament tension in the cell (Grabski *et al* 1994, and M Schindler, Michigan State University, pers comm).

1.11 Transduction of environmental information via modulation of cytoskeleton tension.

In animal cells, changes in cytoskeleton organization are a ubiquitous response to mechanical perturbation (Wang *et al* 1993, Ingber and Folkman 1989, Hay and Svoboda 1989). Microfilaments, microtubules and cytoskeletal regulatory proteins in animal cells have shown changes in

their organization following exposure to growth regulatory molecules (Ingber *et al* 1987), calcium and polyphosphoinositide cascades (Stossel 1993) and lipophilic signalling molecules (Shariff and Luna 1992, Luna and Hitt 1992). Phospholipid metabolites involved in cell signalling pathways have been associated with enhanced intracellular availability of free Ca^{2+} (Berridge and Irvine 1989).

In plant cells, an increase in intracellular calcium (Pasternak and Elson 1985, Grabinski *et al* 1994), magnesium or aluminium (Grabski *et al* 1995) has been shown to increase the tension in actin filaments. Calcium fluxes have been implicated in the anaerobic signal transduction mechanism (Subbaiah *et al* 1994) and induction of touch-responsive genes (Braam and Davies 1990, Trewavas and Knight 1994).

Incubation of plant cells with cytochalasin D (Traas *et al* 1987) or culturing at 4 °C (Marc *et al* 1989) causes a decrease in tension in cytoskeleton transvacuolar strands. Acidification of the cytoplasm of soybean root cells resulted in a decrease in the tension of the cytoplasmic strands as did the application of linoleic or arachidonic acid (Grabinski *et al* 1994). Schindler and co-workers have also treated plant cell cultures with

butyric acid and observed a decrease in actin filament tension (M Schindler, Michigan State University, pers comm).

1.12 Effect of short-chain fatty acids (SCFAs) on plant cell physiology

Hyperosmotic treatment of tobacco suspensions cultures has been shown to result in an initial acidification of the cytoplasm (Renveni *et al* 1987). Acidification of the cytoplasm can be achieved by the use of SCFAs such as butyric acid which rapidly enters the cell and dissociates to protons and butyrate ions, causing the internal pH to fall (Reid *et al* 1989, Smith and Reid 1989). Exposure of plant cells to weak organic acids such as citric acid and an external acid pH also results in a decrease in cytoplasmic pH (Brummer *et al* 1984).

SCFAs are lipid soluble and partition into the lipid phase of the plasma membrane and at least partly remain there. Yuli and Oplatka (1987) have shown that mammalian neutrophil cells undergo considerable fluidization of their plasma membrane when treated with 10 mM propionate.

Butyrate and propionate have been shown in animal systems to induce histone modifications, specifically histone

acetylation due to inhibition of histone deacylases (Leder and Leder 1975, Candido *et al* 1978, Riggs *et al* 1977, Annunziato *et al* 1988). Several studies have shown that millimolar amounts of these organic acids were sufficient to increase core histone acetylation, enhance cell differentiation and to raise overall gene expression (Oliva *et al* 1990) due to chromatin decondensation. Application of fatty acids such as butyrate to *Drosophila* embryos and larvae results in the suppression of position-effect variegation affecting the W+ gene (Mottus *et al* 1980). Arfmann and Haase (1981) demonstrated increased acetylation of histone H4 due to treatment of tobacco suspension cells with butyrate. However, other researchers have been unable to show an increase in histone acetylation in petunia, *Petunia hybrida*, (ten Lohuis *et al* 1995), alfalfa, *Medicago sativa*, (Waterborg *et al* 1990) or artichoke, *Helianthus tuberosus*, (Pederson and Minocha 1988) with butyrate treatment. Ten Lohuis *et al* (1995) found that treatment of petunia cuttings with either butyrate or propionate resulted in a marked increase in transgene methylation levels, but only in a small sub-set of the transgenic population. This was interpreted to mean that site of gene integration was a contributing factor to gene susceptibility to increased methylation and subsequent down regulation.

1.13 Transcriptionally active chromatin is decondensed and therefore open for regulatory transcription-factor binding.

There is evidence that chromatin structure is a factor in the regulation of transcription in plants (Loer and Spiker 1992) as it is in other eukaryotic organisms (Kamakaka and Thomas 1990). Transcriptionally competent chromatin is less compact and more accessible than non-transcribed DNA, but may not be actively transcribed until specific trans-activating factors are present (Kahl *et al* 1987).

1.14 Specific trans-acting factors involved in the regulation of many ABA- and drought-induced genes.

CDeT27-45 promoter nucleotide sequence (Figure 1.1) was examined for putative *cis*-acting promoter elements similar to those found in ABA-inducible genes (Guiltinan *et al* 1990).

Figure 1.1 CDeT27-45 promoter nucleotide sequence[1]

-1226 TGGCATGCTTCTTTGGACACAGAATATTCGTAGATAAGTGTTTATAACATTACCAGAATA -1207

-1206 GTTGACTTTGGGAACAAGCACACGATAGTTTAGAGACTCTGTGATGATATCTCCAAGAGA -1147

-1146 TGTTATTGCAAGTCAATTTTTAAGTAGTGAACCACTTTTGTCTTCGTAGGTCCTTGCTAA -1087

-1086 GGGCACTAGACAAGAGACTATATCTTCTTATTTACGGTGATGCATATGGATCCAAAGGCA -1027

-1026 GCCCAGTTTGGCACTTCCCTGAGAAAGTATACGAATCTGAACAGACACTGCGAAAGGTAA -967

-966 CACTCTTCGCTCCCTTTATTTTGAGTAATTTAAGTTATGCAATTGTCAAATGAACACTGT -907

-906 TTTCACACAATAATGGTTGTTCATCGAATATTGTGTAGTGTGCTGAATCTGCCTTAAAAT -847

-846 CTGTAATCGGAGATCTCTCCCATACTTATTTCGTTGGAAATGCACAATGGGCCATATGAA -787

-786 GTTACAGCCTTCTGAAGACGATCCCTCTCTTAAGGTAAATCATTTCATTACCAACTGCCC -727

-726 TTAAAAGGCCCTTAAATAAAGAACTGTGCAATTATTCTCTCATTTTTCCAGCGATTCTTT -667

-666 TTCAAATCGCAAGTCATCGCAGCGAACAACTTCAATGTGAAAAAGTGCGAAGATTTCGTG -607

-606 TGGGCGACGAAGGACGAACTGTTGGAGTATTTTCC**CGAGCAAG**CCGCATACCTAAACAAG -547

-546 ATGATCATCAGCTGATTACTACCTCTTGCAACTTTCTCTGAAAACTGCTTCTTTGTCCAA -487

-486 ATATAGATAGATTCTTCCCAGTTTTTCACTGTCCCATGAAAATTTTAAACATTTGCCATT -427

-426 GAGTTTTTCTGTAGGCTGGCGAACCATAATAAATCCATTTGACTTGTTGCTTATGAAACT -367

-366 TATGCAAGCCCAAATTTCACAGCCCGATAACCGACC**CGAGAAG**AGTCCATGGGTATTTCC -307

-306 GGTCGAATCTATCCGAACTTTAAACATAGGTAGGAGGGCTATTATTACCTTCTCTACAGT -247

-246 TTCCTTACACGTTTCACTTCGTAAAAAGCCAACTTGCACCGTCTCGTTTGGGCACGTATG -187

-186 TCAGAATTTAGAAAAAAAAAAAAAGTCAGCAAATTTTTATCTCTGATGACTATATCTCTAA -127

-126 TAATAATCACCGACTTTCATGCTGTAAGTTCCAAGTTTCCATTTTTATGACAGCAAAACA -67

-66 CGTACGAGGTACACGTGTGTCCACAATCAGTCTCCATCCATTCCTATATAACAAGCAGCT -7

-6 CGAGAC

1. Michel *et al* 1993

The sequence motifs high-lighted in bold have imperfect homology with *Em*2 sequence motifs (Table 1.2) and double-underlined sequence motifs indicate CACGT sequence locations.

Table 1.1 Sequence comparison of the promoter region of the gene CDeT27-45 with ABA-regulated genes.

Gene	Position[a]	Sequence	Reference
CDeT27-45	-57	TAC**ACGT**GTGC	Michel *et al* 1993
	-70	AAC**ACGT**ACGA	Michel *et al* 1993
	-196	GGC**ACGT**ATGT	Michel *et al* 1993
	-241	TAC**ACGT**TTCA	Michel *et al* 1993
RAB16A	-182	CGT**ACGT**GGCG	Mundy and Chua 1988
RAB16B	-261	AGT**ACGT**GGCA	Yamaguchi-Shinozaki *et al* 1990
RAB16C	-234	CAT**ACGT**GGCG	Yamaguchi-Shinozaki *et al* 1990
RAB16D	-182	CGT**ACGT**GGCG	Yamaguchi-Shinozaki *et al* 1990
RAB17	-163	CAC**ACGT**CCCG	Vilardell *et al* 1991
	-207	GAG**ACGT**GGCG	Vilardell *et al* 1991
Em1a	-149	GAC**ACGT**GGCG	Marcotte *et al* 1989
Em1b	-94	CAC**ACGT**CCGC	Litts *et al* 1991
LeaD7	-128[b]	AAC**ACGT**GTCA	Baker *et al* 1988
LeaD219	-188[b]	CTT**ACGT**GGAT	Baker *et al* 1988
LeaD34	-164[b]	CTC**ACGT**GTCA	Baker *et al* 1988
DC8	-133	ACC**ACGT**GCC	Franz *et al* 1989
Consensus		$^{A}/_{T}$ $^{C}/_{T}$**ACGT**$^{G}/_{T}$ $^{G}/_{T}$	

[a]Represents 5' end point relative to the determined transcription start.

[b]Position relative to the translation start.

Results reported by Marcotte *et al* (1988, 1989) indicated that sequences in the wheat *Em* gene (*Em*1a:GGACACGTGCGC, *Em*1b: GCACACGTCCGC) were responsive to ABA. However, sequences in CDeT27-45 do not match these sequences (Figure 1.1, Tables 1.1 and 1.2), but there is one CACGTG as well as three other sequence motifs containing the CACGT core. The CDeT25-45 promoter sequence has one sequence motif which shows some homology to the wheat Em2 motif (6/8 bps, Table 1.2). This motif is also found in the rice RAB 16 gene (Mundy and Chua 1988) and the soybean β-conglycin gene (Bray and Beachy 1985). The CACGTG sequence has been shown to be one of the promoter elements regulating ultraviolet light induction of *Antirrhinum majus* chalcone synthase gene and is the binding site for nuclear factor CG-1 which has been found in a variety of plant species including tobacco (Staiger *et al* 1989). The CG-1 binding factor was shown by Staiger *et al* (1991) to be constitutively expressed in tobacco and to consist of several polypeptides from 20-40 kD in size, which suggests that in tobacco, these proteins may be general transcription factors. The CACGTG motif has been also found within the palindromic G-box: CCACGTGG, which is found in many stress-induced genes such as anaerobically-induced *Arabidopsis* alcohol dehydrogenase gene (DeLisle and Ferl 1990). Guiltinan *et al* (1990) demonstrated that a wheat leucine zipper protein (bZIP)

can bind to the ABA-responsive element of the wheat *Em* gene. The *Arabadopsis thaliana* bZIP family of G-box binding factors (GBF1 and GBF2, present in leaves and roots, and GBF3, found mainly in root tissue) have been shown to interact with the CCACGTGG motif in *Arabadopsis rbcS-1A* promoter (Schindler *et al* 1992).

Table 1.2 Features of CDeT27-45 promoter from *Craterostigma plantagenium*.

Feature	From To	Comments
A/T-rich	-186 -67	A/T content 76%
A/T-rich	-486 -367	A/T content 68%
A/T-rich	-966 -847	A/T content 68%
ABA-response elements		
CGAGCAAGC	-571 -564	6/8 bp match with *wheat Em2*: CGAGCAGG
CACGT motifs	-57 -47	TACACGTGTGC
	-70 -59	AACACGTACGA
	-196 -186	GGCACGTATGT
	-241 -231	TACACGTTTCA

1.15 A/T-rich sequences bind protein complexes and are associated with transcriptionally active chromatin.

There have been several reports that A/T-rich plant promoter elements give rise to increased transcriptional activity. For example, an A/T-rich promoter from the French bean β-phaseolin gene promoter, fused to the -90 CaMV 35S promoter, resulted in increased expression of the GUS reporter gene in transgenic tobacco (Bustos *et al* 1989). Also, an A/T-rich region of the pea small subunit of the pea ribulose bisphosphate carboxylase (rbcS-3A) gene promoter (Lam *et al* 1990) and the soybean heat shock promoter Gmhhsp17.5E, has been reported to stimulate transcription when placed 5' to a truncated heterologous promoter (Czarnecka *et al* 1992). In contrast to these reports, Castresana *et al* (1988) identified an A/T-rich element of *N tabacum* chlorophyll a/b binding protein (Cab-E) gene promoter as a negative regulatory element.

Czarnecka *et al* (1992) demonstrated, using competition experiments, that A/T-rich fragments from many genes competed for the same binding proteins. The interactions between AT-rich DNA sequences from plant promoters and binding activities present in nuclear extracts have been

shown to be generally comprised of two different DNA binding activities: one or more high mobility complexes (HMC) and a single low mobility complex (LMC) (Schindler and Cashmore 1990, Czarnecka *et al* 1992). The HMC proteins are small proteins with a high percentage of acidic and basic amino acids, are soluble in trichloroacetic acid and are found in association with transcriptionally-active chromatin as are the larger LMC proteins. Tjaden and Coruzzi (1994) have isolated an AT-rich DNA binding protein from tobacco which may be involved in the formation of LMC.

Inhibition of DNA binding by phosphorylation of the DNA binding proteins is a common mode of regulation (Hunter and Karin 1992). Phosphorylation of LMC from pea extracts results in inhibition of binding with A/T-rich promoter elements from the *Nicotiana plumbaginafolia* CabE promoter (Datta and Cashmore 1989). Similar results with soybean extract and the soybean heat shock promoter were reported by Gurley *et al* (1993).

The promoter sequence of CDeT27-45 is characterized by a high level of A/T base pairs. There are three stretches of particularly high A/T content (A/T rich, Table 1.2). The high content of these regions is characteristic of other plant genes and similar A/T-rich sequences of the

β-phaseolin gene linked to *GUS* have recently been shown to direct the expression of a reporter gene in a number of different tissues in transgenic tobacco (Bustos *et al* 1989). Marcotte *et al* (1989) using rice protoplasts, showed that deletion of this region from the *Em* promoter resulted in about a 4-fold decrease in expression of the linked reporter gene *GUS*. The *Em* promoter did, however, still respond to ABA by increasing *GUS* expression greater than 15-fold, indicating that other ABA-inducible sequences were present.

Certainly one of the challenges in sorting out signal transduction pathways is establishing which events may be co-incident but may not be contributing to a particular signal mechanism, from those which are part of the signal transduction mechanism. The induction of CDeT27-45 promoter in response to hyperosmotic stress presented an easily measurable end-point to a signal transduction pathway. My goal was to elucidate some of the critical steps in this signal transduction mechanism.

2 *In vitro* Analysis of Luciferase Expression in Tobacco mediated by a Drought- and ABA-Inducible Promoter from Desiccation Tolerant *Craterostigma plantagineum*

2.1 Introduction

Plant responses to environmental stress such as drought have been extensively studied (Rhodes *et al* 1986), but the mechanisms by which plant cells perceive water deficit and transduce this information into biochemical and molecular responses are not understood. As detailed in the Introduction section, the phytohormone abscisic acid (ABA) is known to play a key role in the drought tolerance of plants (Zeevaart and Creelman 1988).

The desiccation-tolerant *Craterostigma plantagineum* is remarkable in its ability to withstand complete desiccation of its foliage and yet resume viability a few hours after rehydration (Gaff 1971). Specific genes are transcriptionally activated in *Craterostigma* in response to desiccation or application of ABA (Bartels *et al* 1990).

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Five desiccation-induced genes have been isolated and characterized (Piatkowski *et al* 1990). The products of these genes are of great interest because they may be involved in the survival of *Craterostigma* under severe drought conditions. Therefore, in order to study the regulation of these genes, it was necessary to analyse their promoters.

A two kb DNA fragment from the upstream sequence of one desiccation- and ABA-induced gene (pcC27-45) and its 5'-truncated derivatives were transcriptionally fused to the bacterial luciferase reporter fusion gene (*LuxF*) to generate a series of CDeT-45 (*C*raterostigma Desiccation-tolerant) chimeric genes. These genes were used to study promoter activation in response to drought-stress and ABA (Michel *et al* 1993).

The reporter gene used in the experiments reported here was *LuxF* which encodes a monomeric luciferase protein derived from the fusion of *luxA* and *luxB* genes from *Vibrio harveyi* (Escher *et al* 1989). Bacterial luciferases catalyse the oxidation of reduced FMN and long chain aldehydes such as decanal with molecular oxygen to yield FMN, H_2O, the corresponding carboxylic acid and blue-green light (490nm) (Ziegler and Baldwin 1981). Decanal readily penetrates living cells, which permits *in vivo* as well as

in vitro analysis of luciferase activity (Langridge *et al* 1989). Expression of bioluminescence catalyzed by the luciferase enzyme can be monitored in plant tissue *in vitro* or *in vivo*.(O'Kane *et al* 1988).

Two methods that are commonly used to study promoter activation are the transient gene expression (TGE) assay and *Agrobacterium*-mediated plant cell transformation.

TGE in plant cells involves the introduction of genes transiently into plant protoplasts by electroporation, polyethylene glycol treatment or bombardment, and the expression of the introduced gene is evaluated, usually within 24-48 hr. As there is no control over how many plasmid molecules reach the nucleus, results are expressed per number of protoplasts used in the assay. Usually the plasmid DNA is CsCl-purified so that covalently closed circular DNA free of bound proteins is introduced into the protoplast preparation. The majority of the transferred DNA remains extrachromosomal during the time course of the TGE assay (Werr and Lorz 1986) so the analysis is not compromised by influences exerted by chromosomal sequences adjacent to the sites of *trans*-gene integration. In transgenic plants these "position effects" can influence both the level and the specificity of newly introduced genes (Jones *et al* 1985 and Finnegan

and McElroy 1994). On the other hand, loss of the original chromosome environment of the promoter is certainly artifactual and should be kept in mind when considering the observed activity in a heterologous host.

However, TGE assays have been widely used. Regulated expression from a maize alcohol dehydrogenase promoter (Adh1) in maize protoplasts in response to anaerobic stress has been described (Howard *et al* 1987) as has hormonally controlled transient expression of a wheat embryo gene (Em) transformed into protoplasts derived from rice suspension cultures (Marcotte *et al* 1988). Huttly and Baulcombe (1989) using PEG-mediated TGE with cultivated oat aleurone protoplasts, elucidated *cis*-acting regulatory elements in the promoters of wheat α-amylase and other GA_3-responsive genes. The finding that the GA response of the introduced wheat promoter paralleled that of the endogenous oat α-amylase promoter suggested that GA_3-responsive regulation of α-amylase gene expression is not affected by chromosomal structure or DNA methylation and that TGE could duplicate *in vitro* promoter activity.

This chapter describes the expression of full-length and 5'-truncations of CDeT27-45-Lux chimeric genes in tobacco BY-2 cells.

2.2 Materials and Methods

2.2.1 Plasmid constructs

A 2kb 5' region of the CDet27-45 gene containing 1.9 kb of putative promoter was transcriptionally fused to the *LuxF* reporter gene in pPCV699, a pBR322-based binary plant transformation vector pPCV702FP$_2$ derivative (Koncz and Schell 1986, Cohn *et al* 1985, Johnstone *et al* 1986 Figure 2.2.1). 5' CDeT27-45 promoter deletions were generated using exonuclease III digestion and were similarly inserted into pPCV699. Their end-points are shown (Fig 2.2.2) and LTu1 - LTu7 designate *LuxF* fusion products. These plasmids were kindly provided by Dr Dorothea Bartels, Max Planck Institut für Züchtungsforschung, Köln, Germany.

2.2.2 Bacterial strains

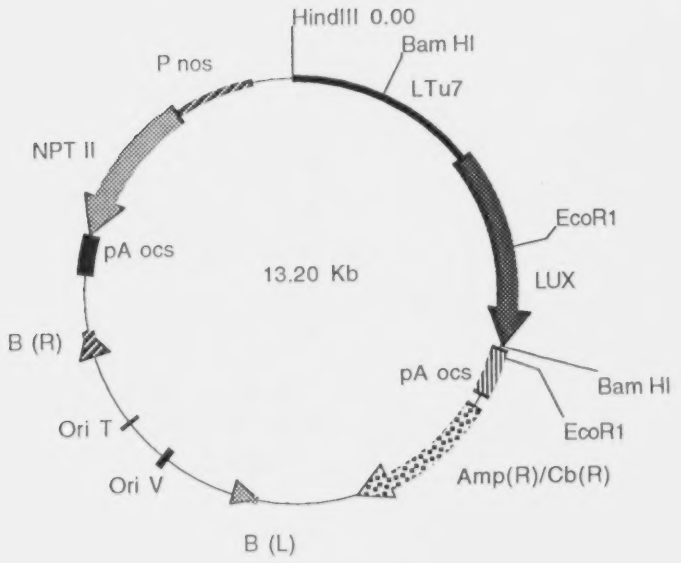
Strains of *Escherichia coli* used for standard amplification of DNA plasmid clones were HB101 and DH5α. (Table 2.2.1). All bacterial strains were grown on Luria broth (LB) medium (1% bactotryptone, 0.5% yeast extract, 0.5% NaCl: 1.2% bactoagar included in solid medium). Strains transformed with pBR322 based vectors were maintained on LB with 100 μg/ml ampicillin.

Table 2.2.1 Bacterial strains used in plant cell transfection/transformation experiments.

Bacterial Strain	Relevant Markers	Medium/selection
E coli HB101	Sm^R, hsdm$^-$, hsdr$^-$, endoI, lacY, leu, pro, thi, rec A	LB
E coli HB101/ pLTu(*LuxF*) plasmids		LB+ Amp^{100}
E coli DH5α	F$^-$, recA1, sup E, Δlac U169, end A1, thi-1, rel A1, hsdr, (phi-80 ΔlacZ-M15)	LB
E coli DH5α/ pLTu(*LuxF*) plasmids		LB+ Amp^{100}

Abbreviations: See List of Abbreviations.

Figure 2.2.1 *Agrobacterium tumefaciens* binary plant transformation vector containing LTu7-*LuxF* insert.

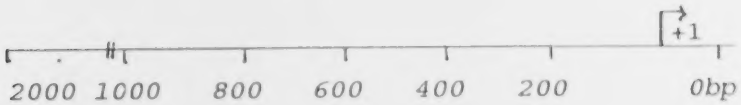


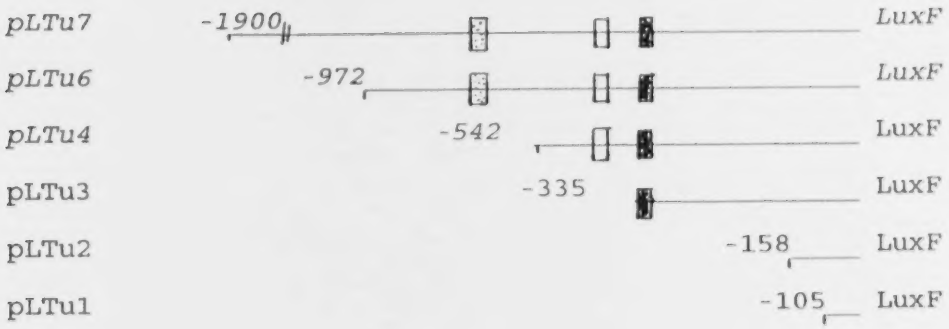
Plasmid name: pPCV(LTu7)LuxF
Plasmid size: 13.20 kb
Constructed by: Koncz and Schell
Construction date: 1986 MGG 204:383-396
Comments/References: Binary plant transformation vector

Promoter deletions LTu6 to LTu1 were similarly inserted in front of bacterial luciferase LuxF gene.

Abbreviations: See List of Abbreviations.

Figure 2.2.2 Structure of pLTu-*luxF constructs.*





Putative regulatory elements indicated as boxes are:

Craterostigma	sequence homologous motifs	Source
Open:	5'AGCCCA 3' 5'A(A/C/G)CCCA-3'	βconglycin/soybean
Solid:	5'CGAGAAG 3' 5'CGAGCAg-3'	Em2 gene/wheat
Stipled:	5' CGAGCAA-3'	Em2 gene/wheat

2.2.3 Amplification of plasmid DNA

Plasmid DNA was purified using the alkaline lysis procedure as described in Sambrook *et al* (1989). One plasmid-transformed colony was picked and used to inoculate 30ml LB medium containing 100 μg ml^{-1} ampicillin. The culture was grown O/N at 37 °C with shaking. 25-ml of this culture was used to inoculate 500 ml LB plus 100μg ml^{-1} ampicillin in a 2L flask. The culture was incubated at 37 °C with shaking for 2.5 h, then 2.4 ml chloramphenicol solution (34 mgl^{-1} in EtOH) was added and incubation continued O/N. The cells were pelleted by centrifuging at 4000 rpm (Sorval GSA rotor) for 10 min at 4 °C. The pellet was resuspended in ice cold STE (0.1 M NaCl, 10 mM Tris.HCl pH 8, 1 mM EDTA pH 8) and centrifuged as before. The pellet was resuspended in 10 ml solution I (50 mM glucose, 25 mM Tris.HCl pH 8, 10 mM EDTA pH 8) and 1 ml freshly prepared lysozyme (20 mgl^{-1} in 10 mM Tris.HCl) was added and the cells were incubated at room temperature for 5 min. 20 ml freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and thoroughly mixed to give a cleared lysate. 15 ml ice-cold solution III (60 ml 5 M KOAc, 11.5 ml glacial acetic acid, 28.5 ml H_2O) was added, with thorough mixing and incubation on ice for 10 min. The white floccy precipitate which formed was

pelleted by centrifugation and the supernatant filtered through cheesecloth. 0.6 vol isopropanol was added to the filtered supernatant and the solution centrifuged to pellet the plasmid DNA. The pellet was washed with 70% EtOH, centrifuged and allowed to air dry at RT. The plasmid pellet was dissolved in 1X TE pH 8.

2.2.4 CsCl-purification of plasmid DNA

8.7 g CsCl was dissolved in 8.7 ml plasmid solution and 300 µl EtBr (10 $mgml^{-1}$ aqueous filtered stock solution) was added. Plasmid DNA/CsCl- solution was transferred to an ultracentrifuge tube and centrifuged in a VTi rotor in a Beckman ultracentrifuge at 52,000 rpm and 25 °C O/N. To remove the plasmid DNA band, a small gauge needle was used to puncture the sealed tube at the top and an 18 gauge needle attached to a 3-5 ml syringe was used to remove the plasmid band. Water-saturated isobutanol extractions were done until no EtBr was visible in the aqueous phase using UV detection. The DNA was precipitated by adding 1 vol sdH_2O and two volumes cold 95% EtOH. Precipitated DNA was centrifuged for 15 min at 4 °C and the pellet washed three times with 70% EtOH, air dried and resuspended in sd H_2O.

2.2.5 Transient expression experiments

Protoplasts were isolated from *Nicotiana tabacum* suspension cell line BY-2 (Kato *et al* 1972). The culture was grown at 22 °C with constant agitation in MS medium supplemented with 3% sucrose, 1.3 mM KH_2PO_4, 0.55 mM myo-inositol, 3 μM thiamine and 0.2 mgl^{-1} 2,4-dichlorophenoxyacetic acid, pH adjusted to 5.8. The cell line was transferred to new medium every seventh day. Three day old cells (logarithmic growth phase) were used for transfection studies.

Cells were harvested by centrifugation at 70 x g, 4 min. Five ml packed cell volume was digested with 50 ml 0.1% cellulase Y-C, 0.01% pectolyase Y23 (Seishin Pharmaceutical Co, Tokyo, Japan) in 2 mM MES, 5 mM $CaCl_2$, 0.5 mM Na_2HPO_4, 0.4 M mannitol digestion buffer pH 5.5. The protoplasts were pelleted and washed three times with protoplast washing solution (0.4 M mannitol, 25 mM $CaCl_2$) and finally resuspended to give 1 x 10^7 protoplasts per ml. Triplicate 0.3 ml protoplast suspension aliquots were independently transfected with CDeT27-45/*LuxF* chimeric gene plasmid DNA (20 μg) using a Ca-PEG delivery system based on the procedure of Maas and Werr (1989) and Båga *et al* (1991). As a gauge of protoplast viability, an

auxin-driven promoter fused to *LuxF* which had been shown to be strongly induced in protoplasts (Båga et al 1991), was included. A solution of 20 μg CsCl-purified plasmid DNA in 500 μl Ca-PEG (25% polyethylene glycol 3350, 100 mM $Ca(NO_3)_2$, 20 mM MES, 0.4 M mannitol, pH 6.0) was added to the protoplast suspension with gentle swirling, then the mixture was incubated at room temperature for 20 min. The transfected protoplasts were pelleted, washed once with transformation washing solution (0.275 M $Ca(NO_3)_2$, 44 mM mannitol). Protoplasts were collected by centrifugation, resuspended in 5 ml protoplast culture medium (MS supplemented with 0.4 M mannitol, 0.28 M sucrose, 1.3 mM KH_2PO_4, 0.55 mM myo-inositol, 3 μM thiamine, 0.2 mg l^{-1} 2,4-D, pH 5.8) and plated in 6 cm Petri dishes. The protoplasts were incubated at room temperature in the dark for 24 h.

2.2.6 Luminometric assay for luciferase activity

Analysis of luciferase activity was carried out essentially as described by Olsson *et al* (1988). Protoplasts were pelleted, resuspended in 500 μl ice-cold lux buffer (50 mM Na_2HPO_4, pH 7, 50 mM 2-mercaptoethanol, 0.4 M sucrose), then sonicated on ice and placed in a luminometer (Turner TD-20e). Following this, 20 μl 1-decanal substrate, as a sonicated 1:1000 aqueous

dilution, and 500 µl light-reduced FMN in tricine buffer (200mM tricine pH 7, 0.4 M sucrose), were then injected into the luminometer to start the reaction. After a delay of 3 sec, the height of the light peak produced during the subsequent 10 sec was taken as the luciferase activity, where 1 light unit (LU) is equal to 1.2×10^8 photons/sec (Koncz *et al* 1986).

The light emitting luciferase catalysed reaction is as follows:

$$RCHO + O_2 + FMNH_2 \rightarrow RCOOH + FMN + H_2O + h\upsilon \ (490\ nm)$$

in which R is an aliphatic moiety containing at least 7 carbon atoms (decanal has 10 carbons) and $FMNH_2$ is reduced flavin mononucleotide.

2.3 Results

2.3.1 Deletion analysis of an ABA and drought-induced promoter:luciferase reporter gene fusion, by transfection assay in BY-2 protoplast cells.

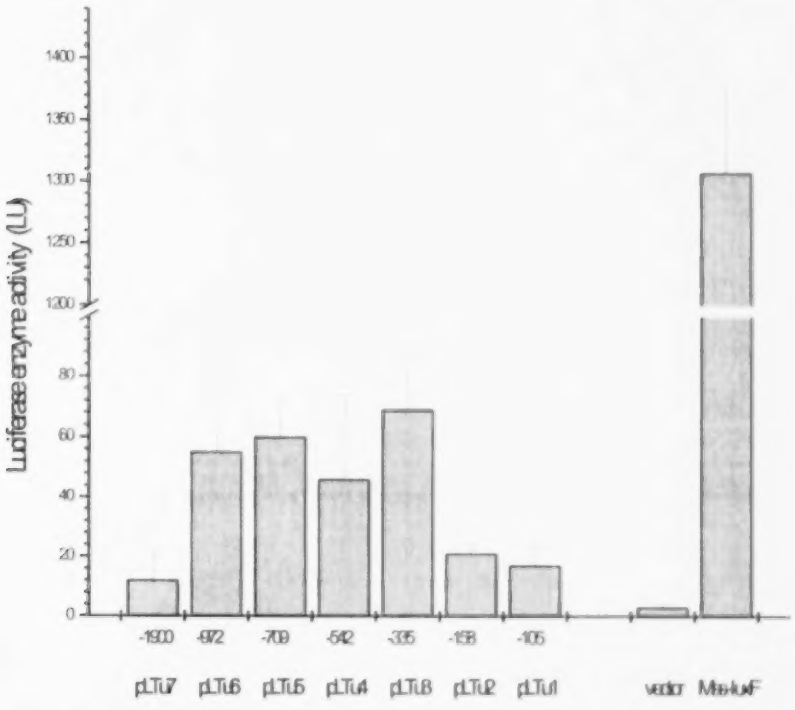
The expression of the CDeT27-45 constructs was monitored *in vitro* by transient gene expression (TGE).

Full-length and 5'-truncated derivatives of the *Craterostigma* pCDeT27-45 promoter, transcriptionally fused to the coding region of the luciferase (*LuxF*) reporter gene to give pLTu-*Lux* chimeric genes (Fig 2.2.1) were obtained from Dr D Bartels (Michel *et al* 1993). These constructs were amplified in *E coli* HB101 and the plasmid DNA extracted and CsCl-purified as outlined in Materials and Methods 2.2.2 and 2.2.3. This plasmid DNA was introduced into tobacco BY-2 protoplasts by the Ca-PEG-mediated transfection protocol for transient expression.

Luciferase enzyme activity generated from 5' truncations of the promoter defines the location of a DNA region essential for significant promoter activation. TGE assays of pLTu-*LuxF* constructs in tobacco protoplasts showed increased luciferase activity with 5'-truncations

of the promoter region to approximately 330 base pairs upstream of the transcriptional start site (construct pLTu3-*LuxF*, Fig 2.3.1). Further deletions reducing the promoter to 158 base pairs (construct pLTu2-*LuxF*) reduced the level of luciferase activity 2-fold. These data indicate that a region involved in promoter activation in tobacco protoplasts is located within or overlapping the 158-335 region. Note that promoter truncations from pLTu6-*LuxF* to pLTu3-*LuxF* gave increased luciferase activity which suggests a negative regulatory element upstream of -972. A possible ABA-inducible function was located on DNA sequences carried by pLTu4-*LuxF*. Incubation of protoplasts transformed with pLTu7-*LuxF* or pLT4-*LuxF* with 10 μM ABA for 7 hr prior to assay, weakly increased luciferase activity a further 2-fold (Figure 2.3.2).

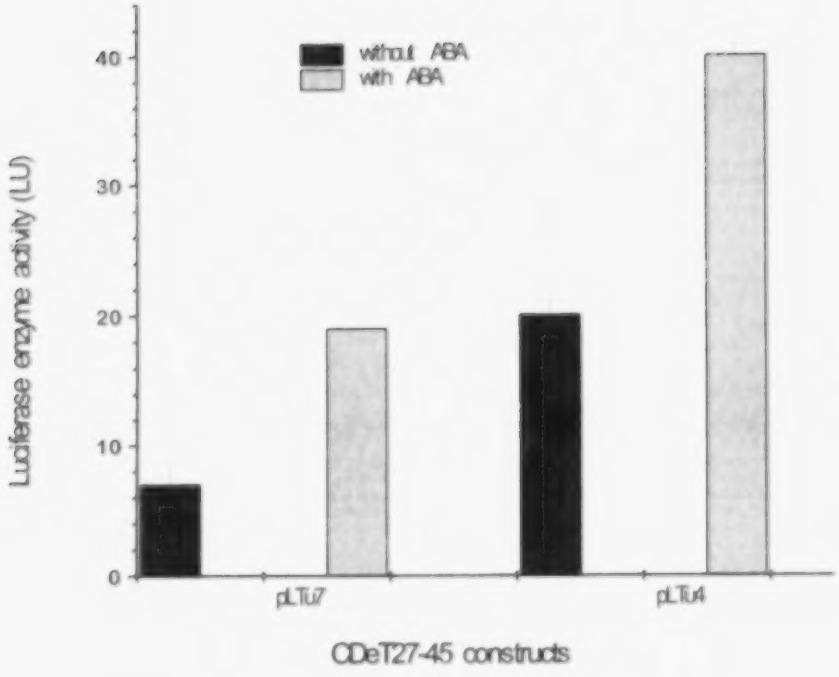
Figure 2.3.1 Luciferase activity generated by CDeT27-45 promoter and 5'-truncations in BY-2 protoplasts.



CDeT27-45 promoter and 5'-deletion end-points.

Each construct shown above was analysed in triplicate. The data shown is from one experiment. This experiment was repeated three times and the overall pattern observed was the same for the three experiments. As outlined in Materials and Methods 2.2.5, the promoterless vector (vector 699) as well as an auxin-regulated vector (*Mas:LuxF*) were included as controls. Luciferase activity was determined after 24 h incubation as described in Materials and Methods 2.2.5 and 2.2.6 and expressed as LU / 10^5 protoplasts.

Figure 2.3.2 The effect of ABA on pLTu4- and pLTu7-induction of luciferase activity.



Protoplasts were transfected with CDeT27-45 constructs pLTu4-*luxF* or pLTu7-*LuxF* as described in Materials and Methods 2.2.5. Luciferase activity was expressed as LU / 10^5 protoplasts (Materials and Methods 2.2.6). 10μM ABA was added 7 h before luminometric assay for luciferase activity. The data shown is from one experiment in which the treatments were analysed in triplicate. This experiment was repeated twice and a similar pattern of response was observed.

2.4 Discussion

Analysis of luciferase gene expression generated by the *Craterostigma* pCDeT27-45 promoter revealed that truncations of the promoter increased luciferase expression suggesting the presence of silencer regions (Dron *et al* 1988, Lawton *et al* 1991) in the sequence upstream from pLTu6. Similar increases in promoter activity in response to initial 5'-deletions have been observed by other researchers. For example, Després *et al* (1995) showed that GUS activity generated by the pathogenesis-related promoter PR-10a in response to wounding or elicitor treatment increased when the PR-10a promoter was truncated from -1015 to -670 bps.

Truncations downstream of pLTu3 resulted in reduced luciferase expression (Figure 2.3.1). The addition of ABA to pLTu4-*luxF* and pLTu7-*luxF* increased luciferase expression only slightly (Figure 2.3.2). However these findings were not observed by our collaborators (Michel *et al* 1993) using either tobacco leaf mesophyll protoplasts or *Craterostigma* callus-derived protoplasts. They observed that CDeT27-45 full-length promoter and 5'-truncations to 282 base pairs required ABA for expression of GUS activity in both systems (Michel *et al* 1993). As we were using protoplasts derived from BY-2 tobacco cell

suspension culture, it may be that this cell line has de-differentiated to such an extent that required ABA-responsive transcription factors are not present. Testing a known ABA-responsive promoter such as the *Em* promoter which in rice suspension cells has been shown to be strongly synergistic in response to ABA and salt-induced osmotic stress (Bostock and Quatrano 1992) in the BY-2 protoplast system would have clarified this question. The robust response by protoplasts transfected with the *Mas* promoter to auxin present in the medium (Langridge *et al* 1989) indicated that the protoplast preparations were viable. Expressing luciferase activity generated by CDeT27-45 promoter relative to luciferase activity generated by the *mas* promoter gives values of 1% for the full-length promoter and 3% for the pLTu4.

Acknowledgement

I would like to thank Dr Monica Båga for her instruction and assistance with this research.

3 In vivo deletion analysis of a *Craterostigma* ABA- and drought-induced promoter stably transformed into *Nicotiana tabacum*.

3.1 Introduction

To determine if the CDet27-45 promoter confers drought- and ABA-inducible regulation in a heterologous host, CDet27-45-*LuxF* chimeric genes were introduced into *Nicotiana tabacum* by *Agrobacterium tumefaciens*-mediated transformation. Using this method, foreign DNA is stably integrated into the plant chromosome. The plant cell which is thus transformed can now be grown on kanamycin medium and form transformed callus. In the presence of plant growth factors, transformed callus develops shoots which can be removed and placed individually in rooting medium so that complete plants are formed. Luciferase enzyme activity can then be measured in various tissues of primary and self-pollinated, kanamycin-resistant transformed tobacco plants.

This chapter describes the *in vivo* analysis of CDeT27-47 promoter and its 5'truncations in whole tobacco plants.

A version of this chapter has been published. Michel D, Salamini F, Bartels D, Dale P, Båga M, and Szalay AA (1993) Plant J 4, 29-40

3.2 Materials and Methods

All chemicals and prepared media were obtained from the Sigma Chemical Company, St Louis, Missouri, unless otherwise noted.

3.2.1 Plasmid constructs

Plasmid constructs were the same as those used for TGE assay and are described in Chapter 2, Materials and Methods 2.2.1 and Figure 2.2.1. *E coli* strain S17.1, transformed with CDeT27-45*LuxF* constructs as outlined in Section 3.2.2, was used to conjugally transfer these constructs, as outlined in Section 3.2.3, to *Agrobacterium tumefaciens*, a binary plant vector described in Table 3.2.1.

3.2.2 Preparation and transformation of competent *E coli* S17.1 cells.

Competent *E coli* cells were prepared using the method of Chung *et al* 1989. An O/N culture of *E coli* strain S17.1 was diluted 1:100 in 10 ml LB medium (per L: tryptone 10g, yeast extract 5.0g, NaCl 10g) and grown at 37 °C with shaking to an OD_{600} 0.3-0.4. The cells were pelleted as 1 ml aliquots in Eppendorf tubes, by centrifugation at 6000

rpm for 5 min and resuspended in 0.1 ml TSS medium (LB medium pH 6.5 containing 10% polyethylene glycol (PEG) 3350, 5% dimethylsulfoxide (DMSO) and 50 mM $MgCl_2$). The cells were mixed with an equal volume of ice-cold TSS and held on ice 20 min. 100 ng plasmid DNA was added to one tube of competent *E coli* S17.1 and the cells were held on ice 5 min before 0.9ml TSS containing 20mM glucose was added. The cells were incubated at 37°C for 60 min, with vigorous shaking. 10 to 100 µl aliquots were plated on LB agar (LB medium plus 15g/L agar) containing 100 µg/ml ampicillin.

Table 3.2.1 Bacterial strains used in plant cell transformation experiments.

Bacterial Strain	Relevant Markers	Medium/selection
E coli S17-1	RP 4-2 (Tc::Mu), (Km::T7), Sm, Pro, hsdr, recA	LB
E coli S17-1/ pLTu(*LuxF*) plasmids		LB+ Amp^{100}
A tumefaciens GV3101(pMP90RK)	Rif^R recipient strain containing plasmid pMP90 (Gm^R), a deletion derivative of Ti-plasmid pTiC58 containing plasmid pBR322 sequences and RK2013(Km^R) encoding helper (tra)functions permitting conjugal transfer of plasmid DNA from *E coli* to *Agrobacterium*.	YEB+Mg^{2+}, Rif^{100}, Km^{25}, Gm^{25}
A tumefaciens GV3101(pMP90RK)/ pLTU(*LuxF*) plasmids		YEB+Mg^{2+}, Rif^{100}, Km^{25}, Gm^{25}, Cb^{100}

Abbreviations: See List of Abbreviations.

3.2.3 Transfer of pCDeT27-47*LuxF* plasmid DNA from *E coli S17.1* to *Agrobacterium tumefaciens*.

The LTu promoter-luxF fusions inserted in the binary vector pPCV699 (Figure 2.2.1, Koncz and Schell 1986, Cohn *et al* 1985, Johnstone *et al* 1986) and carried by the mobilizing *E. coli* strain S17-1 (Simon *et al* 1983) were transferred to *Agrobacterium tumefaciens* strain GV3101/pMP90RK by conjugation using the following protocol. *E coli* S17.1 transformed with CDeT27-45*LuxF* plasmid DNA as outlined above was grown as the donor strain on YEB agar plates (per L: beef extract 5 g, yeast extract 1 g, peptone 1 g, sucrose 5 g and 1.5% agar) containing $MgSO_4$ (10 mM) and ampicillin (100 mgl^{-1}). *Agrobacterium tumefaciens* strain GV3101/pMP90RK as the recipient strain was grown on YEB medium containing $MgSO_4$ (10 mM), rifampicin (100 mgl^{-1}), kanamycin (25 mgl^{-1}), gentamycin (25 mgl^{-1}). Equal amounts of donor and recipient strains were scraped off the plates and resuspended in sterile polypropylene tubes in 5 ml YEB liquid medium containing $MgSO_4$ (10 mM). The cells were pelleted by a brief centrifugation at 4000 rpm and resuspended in YEB liquid medium containing $MgSO_4$ (10 mM) to give 2x 10^8 cells in 100 μl for *A tumefaciens* and 6x 10^8 cells in 100 μl for *E coli* as determined by measuring

OD_{550} and calculating the appropriate dilution based on the conversion: 1 OD_{550}= 6x 10^8 cells/ml. Aliquots of 50-100 μl *E coli* and *A tumefaciens* were each applied to the same YEB plus $MgSO_4$ (10 mM) agar plate and mixed while spreading. The plates were incubated at 29 °C for 24 h. Colonies were scraped off these plates and resuspended in 1 ml $MgSO_4$ (10 mM). OD_{550} was measured and the cells diluted to give 5x 10^8 cells per 100 μl. Dilutions (5x 10^7, 1x 10^6, 5x 10^8) were spread on YEB plus $MgCl_2$ agar selection plates containing kanamycin (50 mgl^{-1}), gentamycin (25 mgl^{-1}) and carbenicillin (100 mgl^{-1}). Donor and recipient strains were also plated separately on these selection plates, as a check on selection stringency. Selection plates were incubated at 29 °C for 24 h. Single *A tumefaciens* colonies were picked from the selection plates and restreaked for single colonies on selection plates. Culture material from single colonies which had been grown as a patch was mixed in lux buffer and assayed for luciferase activity as outlined in Materials and Methods 2.2.5.

3.2.4 *Agrobacterium tumefaciens*-mediated leaf explant transformation procedure.

Leaf discs of *Nicotiana tabacum* (cv Petit Havana SR1) were transformed as described by Horsch *et al* (1985).

Agrobacterium tumefaciens transconjugants resistant to carbenicillin (100 mg l^{-1}), kanamycin (25 mg l^{-1}), gentamycin (25 mg l^{-1}) and rifampicin (100 mg l^{-1}) were selected and used for leaf disc transformation. Plasmid integrity was verified by restriction enzyme analysis of plasmid DNA isolated from *E coli* which had been transformed by cocultivation with *Agrobacterium tumefaciens* transconjugants. The *Agrobacterium* transconjugant-infected leaf discs were incubated on solid MS medium for two days and subsequently transferred to MS agar medium supplemented with 1.0 mg l^{-1} 6-benzylaminopurine, 0.1 mg l^{-1} naphthalene-acetic acid, 100 mg l^{-1} kanamycin (for selection of kanamycin-resistant shoots) and 200 mg l^{-1} claforan (for deselection of CDeT27-45*LuxF*-transformed *Agrobacterium*). Shoots were rooted on MS agar medium in the presence of kanamycin and claforan and rooted plantlets were transferred to soil and grown in a greenhouse.

3.2.5 Osmotic stress, ABA and drought treatment of kanamycin resistant plants

Plants which had rooted on 100 mgl^{-1} kanamycin were transferred to soil and grown in a greenhouse. Flower heads were bagged before opening to insure selfing. Seed was collected and sterilized by treatment with 70%

ethanol (2 min), 10% commercial bleach plus 0.1% tritonX-100 solution (10 min), rinsed 5 times with sterile distilled water and air dried on sterile filter paper. Seed was germinated on MS agar plates containing 100 mgl^{-1} kanamycin before transfer to soil. Leaf discs from these kanamycin-resistant plants were floated in solution containing 0.7 M mannitol as the hyperosmotic stress, with or without 100 μM ABA or in water, with or without ABA as above. Plants were also droughted and leaf discs excised from wilted leaves.

3.2.6 Luminometric assay for luciferase activity

Analysis of luciferase activity was carried out essentially as described by Olsson *et al* (1988). Tobacco leaf discs cut with an 8 mm metal test-tube cap, from a young, fully expanded leaf, were ground in an Eppendorf tube in 500 μl ice-cold lux buffer (50 mM Na_2HPO_4, pH 7, 50 mM 2-mercaptoethanol, 0.4 M sucrose), and maintained on ice. 500 μL of cleared (by settling) homogenate were placed in a luminometer (Turner TD-20e). Following this, decanal and reduced FMN were added as substrates for luciferase enzyme reaction as described in Chapter 2, Materials and Methods 2.2.5.

Protein concentration of cleared supernatant was

determined according to the method of Bradford (1976), using dilutions of BSA (1 mg/ml) stock solution as protein standards.

3.2.7 Plant Genomic DNA Extraction

Plant DNA was extracted essentially as described by Doyle and Doyle (1990). Fifteen mls of CTAB extraction buffer (2% CTAB, 100 mM Tris.HCl pH 8, 20 mM EDTA pH 8, 1.4 M NaCl, 1% PVP (polyvinylpyrrolidone) Mw 360,000), pre-heated to 60-70 °C, was added per gram of plant leaf tissue, which was first ground to a fine powder in liquid nitrogen with a mortar and pestle. The powdered tissue was transferred to the pre-heated CTAB, gently mixed by inversion, then incubated in an 60 °C bath for 15 min with occasional mixing. One volume chloroform/isoamyl alcohol (24:1) was added and gently mixed by inversion over several minutes. The phases were separated by centrifugation at 10,000 rpm for 10 min in a Sorvall S-600 rotor. The aqueous phase was transferred to a fresh tube using a wide-bore pipette. If the supernatant was cloudy, the chloroform-isoamyl alcohol extraction was repeated. A 2/3 volume of isopropanol was added to the clear supernatant and the precipitated genomic DNA was spooled onto a glass pipette. The spooled DNA was washed by immersion in two changes of wash buffer (75% EtOH, 10

mM ammonium acetate). The DNA was lightly pelleted by slow centrifugation and the ethanol removed by air drying at RT. The DNA was finally dissolved in 1X TE (10 mM Tris.HCl pH 8, 1 mM EDTA pH 8) at 4 °C overnight.

3.2.8 Southern Transfer Procedure

Genomic DNA (10 μg) was digested with the indicated restriction enzymes (5 Units enzyme per μg DNA for 3 h at 37oC) and separated on a 0.8% agarose gel in 1X TBE buffer (89 mM Tris-borate, 2 mM Na-EDTA pH 8). The agarose gel was soaked for 15 min in 0.25 M HCl, rinsed in 2X SSC (300 mM NaCl, 30 mM trisodium citrate), then incubated in denaturation buffer (1.5 M NaCl, 0.5 M NaOH) with gentle agitation for 30 min and finally incubated in neutralization buffer (1.5 M NaCl, 0.5 M Tris.HCl pH 7.2, 1 mM EDTA) for 30 min. DNA was transferred by capillary blot with 20X SSC (3 M NaCl, 300 mM trisodium citrate) onto Hybond N membranes (Amersham). The Southern blot was prehybridized 4 h at 65oC, in 6X SSC (900 mM NaCl, 90 mM trisodium citrate), 5X Denhardt's solution (0.1% Ficoll 400,000 MW, 0.1% PVP 360,000 MW, 0.1% BSA), 0.5% SDS, with 10 mg denatured herring sperm DNA. The purified random labelled probe was denatured by heating in a boiling water-bath for 5 min, before addition to the existing prehybridization buffer. Hybridization was

continued at 65 °C for at least 16 h. The membrane was washed twice in 2X SSC, 0.5% SDS at RT for 15 min and then once with 1X SSC, 0.1% SDS at 65 °C for 15 min, then exposed at -80 °C to X-ray film (Kodak X-OMAT) in a cassette with an intensifying screen.

3.2.9 Probe Labelling

One-Kb LuxF SalI-EcoRI fragment used for random labelling was first purified from agarose gel using glass powder. Fifty micrograms of the desired DNA fragment was made up to 31 μl with sd H_2O and denatured in a boiling water-bath for 10 min. The DNA was cooled on ice for 5 min, then added to 10 μl oligo-labelling buffer (Pharmacia Random Hexamer labelling kit), 100 μM d(A,G or T)TP, 5 mM β-mercaptoethanol, 0.2 OD units random hexamer oligonucleotides and 2 μg BSA. The labelling reaction was initiated by adding 50 μCi of a α-[^{32}P]-dCTP and 2 units of *E coli* DNA polymerase Klenow fragment (Pharmacia). The reaction was mixed well and allowed to proceed for 5 h. Labelled fragments were purified from unincorporated α-[^{32}P]-dCTP by passage down a Sephadex G-50 column and a 1 μl labelled-fragment aliquot was removed for Cherenkov counting in a Beckman scintillation counter.

3.3 Results

A total of ninety-five kanamycin-resistant tobacco plants for all constructs (Figure 2.2.2.) were generated using *Agrobacterium*-mediated plant transformation protocol and of these transformants only nine demonstrated luciferase activity. No lux activity was detected in the leaf tissue, roots or flowers of any of the kanamycin-resistant CDeT27-45 transformants (Table 3.3.1). Lux activity was highly expressed in mature seeds of plants transformed with LTu 4 and longer constructs (Table 3.3.1). Values in Table 3.3.1 are the maximum levels of luciferase activity per ng total soluble protein obtained for each particular construct. Figure 3.3.1 indicates the luciferase activity per cm^2 leaf tissue obtained for each luciferase-expressing transformant.

However, luciferase activity was induced in pLTu7-*luxF*, pLTu6-*luxF* and pLTu4-*luxF* transformed leaf tissue immersed in a non-penetrant osmoticum such as 0.7 M mannitol solution (Table 3.3.1). With the exception of one transformant, LTu4.5, similar levels of luciferase activity were obtained from each transformant in response to osmoticum.

Table 3.3.1 Specific Luciferase activity in tissues of transgenic tobacco

Construct/5' end point	number of plants[b]	LU ng^{-1} protein[a] Seeds	Leaves/Roots/Petals[c] -ABA, +ABA, wilting	Leaves osmoticum
pLTu1/-105	20		<10	<10
pLTu2/-158	13	<10	<10	<10
pLTu3/-335	21	<10	<10	<10
pLTu4/-542	9(3)	1000	<10	(3) 150
pLTu6/-972	14(3)	400	<10	(3) 50
pLTu7/-1900	8(3)	400	<10	(3) 50
pPCV699B-luxF[d]	10	<10	<10	<10

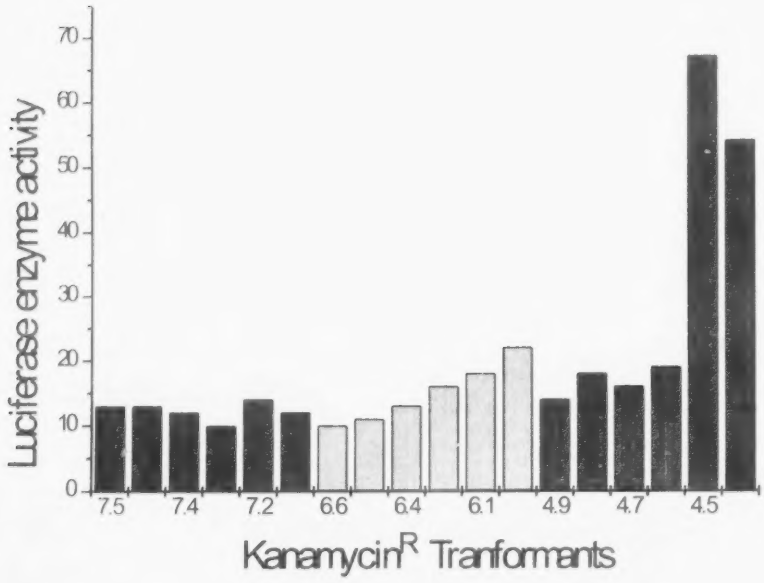
a. Luciferase activities are expressed as LU ng^{-1} protein where 1 LU is equal to 1.2×10^{8} photons sec^{-1}. Activity levels indistinguishable from background are indicated as <10.

b. The number of independent shoots that rooted and grew in the presence of 100 μg ml^{-1} kanamycin. Numbers in parentheses indicate numbers of plants generated from kanamycin resistant seed that expressed detectable levels of bioluminescence and the maximum level expressed by that construct is given.

c. Leaf discs, root sections and flower petals were floated in solution containing 0.7M mannitol without (-ABA) or with 100μM ABA (+ABA) for 24h or plants were droughted to the point where they were visibly wilted (wilting) as described in Materials and Methods 3.2.5.

d. pPCV699B-luxF is the promoterless vector.

Figure 3.3.1 Luciferase activity induced by 0.7M mannitol in CDeT27-45:luxF transformed kanamycin-resistant transgenic tobacco plants.

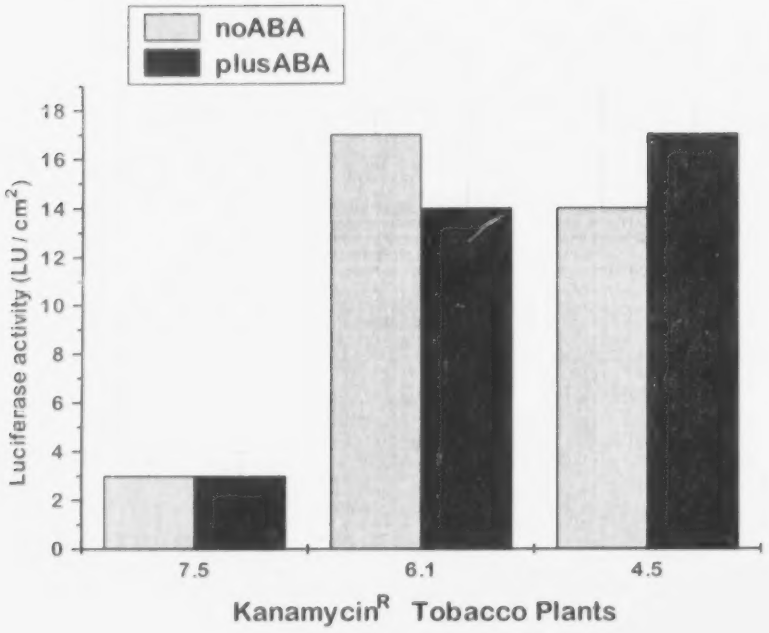


Leaf sections were excised from young, fully expanded leaves of selfed, kanamycin-resistant CDeT27-45-*luxF* transformed tobacco plants. These sections were immersed in 0.7 M mannitol for 24 h before luminometric assay for luciferase activity. The above graph shows two independent determinations of luciferase enzyme activity, given as LU / cm ^{2}, in kanamycin resistant tobacco transformants. 7.2, 7.4 and 7.5 refer to kanamycin-resistant plants which grew out of independent calli on leaf discs which had been co-incubated with LTu7-transformed *Agrobacterium*. These shoots were resistant to 100 μg/ml kanamycin. The same numbering format applies to shoots obtained from LTu6- and LTu4-transformed calli. A similar pattern was always observed among the transformants expressing kanamycin resistance and luciferase enzyme activity.

The values in Table 3.3.1 represent the maximum level of

luciferase activity from independent kanamycin-resistant transformants for each construct. The presence of 100 μM ABA did not change the levels of osmotically induced luciferase enzyme activity (Figure 3.3.2).

Figure 3.3.2 The effect of 100 μM ABA on osmotic induction of luciferase activity.



Leaf sections were excised from young, fully expanded leaves of transformants LTu7.5, LTu6.1 and LTu4.5 and immersed in 0.7M + 100μM ABA for 24 h before luminometer assay for luciferase enzyme activity (LU/cm^2) as outlined in materials and methods 2.2.6. The values shown represent an average and standard deviation among 4 luciferase activity assays per transformant.

Figure 3.3.3 shows a Southern transfer of total genomic DNA isolated from kanamycin-resistant, luciferase activity positive transformant 7.5 and digested with restriction enzymes Bam H1 and EcoR1. The transfer was probed with ^{32}P-labelled 1 kb *LuxF* fragment. Bam H1 cuts within CDeT27-45 promoter at -900 bp and at the end of the *LuxF gene*, releasing the 3 kb fragment while Eco R1 cuts in the middle of the *LuxF* fragment as well as just within the polyadenylation sequence, pA ocs, of the luciferase plant transformation cassette, resulting in the release of a 1 kb fragment (Figure 2.2.1). As a marker, a Bam H1-digested, ^{32}P-labelled 3 kb *LuxF* + promoter fragment of pLTu7 was also run on this gel. This Southern showed the expected 3 kb fragment released by Bam H1 digestion and the 1 kb fragment released by Eco R1 digestion which indicates that the luciferase plant transformation cassette was incorporated into the tobacco genome of transformant 7.5. There appear to be other sequences to which the *LuxF*-probe was hybridizing. This was most likely due to incomplete digestion of the genomic DNA as indicated by the smearing at the top of the lanes.

Figure 3.3.3 Autoradiograph of a Southern transfer of tobacco transformant 7.5 genomic DNA probed with *LuxF* ^{32}P-labelled fragment.

Lanes 1 - 3 and 4 - 6 are Bam H1 and EcoR1 digests respectively, of 10, 5, 3 μg transformant 7.5 genomic DNA. Lux Marker was ^{32}P-labelled 3 kb promoter + *LuxF* fragment.

3.4 Discussion

Signal transduction pathways leading to gene expression in response to perceived environmental stress have been found to operate via several mechanisms. Certainly the phytohormone ABA is important in plant response to drought, salinity and cold stress (Singh *et al* 1989, Zeevaart and Creelman 1988). ABA levels have been shown to change in a number of plant species during seed development (Finkelstein *et al* 1985, Galau *et al* 1986) and ABA has been shown to regulate the synthesis of some seed storage proteins (Bray and Beachy 1985, Williamson *et al* 1985). In developing cotton seeds, a set of late embryogenesis-abundant mRNAs (*Lea* mRNA) is induced by ABA (Galau *et al* 1986) and a number of *Lea* genes have been cloned from other plant species (Dure *et al* 1989, Curry *et al* 1991).

Specific ABA responsive elements (ABREs) have been identified in promoter regions of ABA-inducible genes (Table 1.1, Mundy *et al* 1990). Comparison of these sequences with CDeT27-45 revealed that four related motifs are present at -57,-70, -196 and -241 relative to the transcription start site (Introduction, Table 1.2). Common to all these sequences is the core motif of the G-box (C**ACGT**G) which has been identified in the 5' upstream

region of plant genes regulated by a variety of environmental signals and physiological cues (Williams *et al* 1992). For example, the light-responsive chalcone synthase promoter of *Antirrhinum majus* also contains the CACGTG motif (Staiger *et al* 1989) which suggests that unique factors, which recognize similar sequences, respond via different mechanisms to a particular stress or environmental cue.

In transgenic tobacco, the CDeT27-45 promoter directs expression in seed (Table 3.3.1). Thus the CDeT27-45 promoter is tissue-specific and its expression is confined to the embryo of the maturing seed at a time when the seed is dehydrating (Table 3.3.1). Therefore it may be that the increasing osmoticum is the inductive mechanism in maturing seed as well.

The CDeT27-45 gene is silent in vegetative tissues of the resurrection plant *Craterostigma plantagineum* but is activated in these tissues by ABA or desiccation to synthesize CdeT27-45 protein (DSP 15, Group 4 *Lea*, Introduction 1.2) in leaves and roots as well as in callus (Bartels *et al* 1990). Similarly, CDeT27-45 was found to be silent in vegetative tissues of CDeT27-45*LuxF*-transformed tobacco, (Michel *et al* 1993 and Table 3.3.1, this thesis), but was not induced by application of ABA to- or by wilting CDeT27-45 transformed tobacco.

The observation that CDeT27-45*GUS* was responsive to ABA in transfection studies using tobacco protoplasts, while CDeT27-45*GUS*, like CDeT27-45*LuxF*, was unresponsive to ABA in stably transformed tobacco, was puzzling. No synergistic effect between ABA and osmotic induction of luciferase enzyme activity in stably transformed tobacco was observed. The level of luciferase activity induced by osmotic stress was not changed in the presence of ABA (Figure 3.3.2). Taken together, these findings suggest that ABA-regulation of integrated CDeT27-45 promoter does not occur in vegetative tobacco cells. Possibly there is insufficient homology between *Craterostigma* and tobacco ABA-responsive sequences for binding of required *trans*-acting factors in the integrated *trans*-gene or a change in chromatin conformation is required before activation of transcription occurs.

While the CDeT27-45 *luxF* chimeric genes transformed into tobacco do not respond to either wilting or application of ABA (Table 3.3.1), it was observed while making protoplasts from transgenic leaves, using the method of Draper *et al* (1988), that CDeT27-45 could be induced in leaf tissue by hyperosmotic conditions. Luciferase enzyme activity was induced in transformed leaf tissue in response to 0.7 M mannitol (Table 3.3.1) and the level induced was not changed by the addition of exogenous ABA

to the hyperosmotic medium (Figure 3.3.3).

Similar activity patterns have been described for the promoter of the *Em* gene of wheat (Marcotte *et al* 1989) and for the *rab*-16B promoter from rice (Yamaguchi-Shinozaki *et al* 1990). When these promoters were transferred to tobacco in reporter gene constructs, they resulted in GUS expression in embryos but not in vegetative tissue. The maize *rab*-17 gene promoter also exhibited high activity in tobacco embryos and low activity in osmotically stressed vegetative tobacco tissue (Vilardell *et al* 1991).

The pattern of luciferase enzyme activity in response to osmotic stress paralleled the pattern observed in seeds: only transgenics containing constructs longer than LTu3/-335 responded to hyperosmotic conditions, with maximum luciferase activity observed from one of the LTu4 transformants. This may be the result of integration into a more transcriptionally active chromatin domain i.e. position effect (Finnegan and McElroy 1994). The observation that despite promoter truncations up to 1358 bases, the level of luciferase enzyme activity induction by osmotic stress was relatively uniform among eight of the nine transformants and was not induced by ABA in any of the transformants, suggests that a more general

promoter activation mechanism may be involved.

CDeT27-45 promoter is somewhat A/T rich (Introduction, Tables 1.1 and 1.3), particularly within -67 to -186 and -367 to -486 of the promoter sequence. As discussed in the Introduction 1.14, A/T-rich sequences bind protein complexes which constitutes a feature associated with transcriptionally active chromatin. The loss of these sequences from promoter-truncations LTu3 and smaller, possibly resulted in the loss of osmotic induction of luciferase enzyme activity.

The lack of ABA response in CDeT27-45-*LuxF* transformed tobacco removes a layer of complexity from the signal transduction mechanism(s). Consequently the CDeT27-45 promoter may be useful in elucidating the mechanisms involved in plant cell recognition of osmotic stress and which lead to the induction of luciferase activity in response to that stress.

Acknowledgement

I would like to thank Dr Monica Båga for her instruction and assistance with this research.

4 Induction of an osmotically-sensitive promoter by short-chain fatty acids.

4.1 Introduction

As outlined in the Introduction section 1.5, plants have developed a variety of signal transduction mechanisms involving ion fluxes: anaerobic stress has been shown to involve Ca^{2+} fluxes (Subbaiah *et al* 1994) as do the signal transduction mechanisms which activate wind- and touch-induced genes (Braam and Davies 1990, Trewavas and Knight 1994). Shifts in intracellular pH have also been proposed to be the second messenger which mediates ABA- and other stress-induced metabolic changes (Kurkdjian and Guern 1989, Guern *et al* 1991).

The induction of CDeT27-45-*LuxF* fusion gene in transgenic tobacco vegetative tissue in response to hyperosmotic stress, but not in response to elevated ABA or drought, suggested that I might be able to focus on those elements that are unique to the osmotic stress signal transduction mechanism.

One of the reported effects of plasmolysis is membrane depolarization (Li and Delrot 1987). This means the ion gradient across the plasma membrane is changed due to an

influx of protons or other ions such as potassium. Acidification of suspension cell medium by plant cells exposed to mannitol and other similar osmotica is well documented (Reinhold *et al* 1984, Reuveni *et al* 1987), but these data record a net efflux that occurs over time due to proton-stimulated H'-ATPase activity (Spickett *et al* 1992). Thus the initial signal in the osmotic signal transduction pathway could be cytosolic acidification which activates pH-dependent proteins and amplifies this stress induced signal.

Treatment of plant cell suspension cultures with weak organic acids such as benzoic acid or citric acid or short-chain fatty acids such as propionate, butyrate and hexanoic acid results in an acidification of the cytoplasm due to the release of protons in the neutral pH of the cytoplasm (Kurkdjian and Guern 1989). Frachisse *et al* (1988) showed that, in *Riccia* cells, acetic, propionic, butyric and hexanoic acids acidify the cytoplasm according to the concentration of their protonated form. In the presence of acetic, propionic and butyric acid, the cytoplasmic pH partly recovers, while hexanoic rapidly depleted the electrochemical pH gradient across the plasma membrane. All of these fatty acids showed a concentration-dependent decrease in membrane resistance which suggests that membrane perturbations

have also occurred.

Yuli and Oplatka (1987) showed that 10 mM propionate acidified the cytoplasm of human neutrophil cells just as chemoattractants do and that both phenomena could be inhibited by proton uncouplers such as FCCP (carbonyl cyanide p-trifluoromethoxyphenylhydrazone). The K^+-ionophore valinomycin did not affect the acidification and these researchers suggest that cytosolic acidification may be the initial step in chemoattractant signal transduction in neutrophil cells. They also showed by fluorescent anisotrophy measurements that there was appreciable membrane fluidization as a result of propionate treatment.

Shifts in plant cell membrane polarization have also been obtained by treating cells with potassium ionophores such as valinomycin (Glaser and Donath 1989) and H^+-ATPase stimulators such as fusicoccin (Johansson *et al* 1993, Blatt and Clint 1989, Reid *et al* 1985). Ionophores such as valinomycin act as ion-specific channels which allow a flux of ions into the cell, thus changing internal concentrations and affecting ion gradients across membranes.

While short-chain fatty acids have been shown to acidify

cytosolic pH in plant cells in a concentration dependent manner (Frachisse *et al* 1988), butyric acid is known to affect chromatin structure in animal cells (Lee *et al* 1993). Mammalian cell histone deacylase is inhibited by butyric acid which results in histone hyperacetylation and consequently chromatin decondensation (Boudreau *et al* 1995). Butyric acid does not affect deacylases in most plant systems tested (ten Lohuis *et al* 1995), although Arfmann and Hasse (1981) presented data which indicated that butyric acid was affecting chromatin structure in tobacco suspension cells. Propionic acid and butyric acid also have been shown to affect DNA methylation pattern in plant cells (ten Lohuis *et al* 1995).

Grabski *et al* (1994) have developed an assay to measure cytoskeleton tension in plant cells using a focused laser beam to optically trap and displace cortical cytoskeleton a defined distance within the cell. They have demonstrated that decreases in pH as well as exogenous application of lipids such as linoleic acid decreased cytoskeletal tension. Treatment of suspension cells with butyric acid also results in a decrease in actin-filament tension (M Schindler, Michigan State University, pers comm).

Since there are a number of events that could be involved

in the osmotic stress induced pathway, in this chapter, I outline the effects of compounds which have been shown to influence parameters such as ion flux and membrane polarization status, on the induction of luciferase activity in cell suspension cultures.

4.2 Materials and Methods

4.2.1 Materials

Unless otherwise stated, all chemicals and plant tissue culture supplies were obtained from Sigma Chemical Company, St Louis, Missouri, USA. Butyrate was added to suspension culture medium from 1 M aqueous stock to give the concentrations cited in figure legends. Other SCFAs tested were similarly prepared as 1M aqueous stocks. Cytochalasin D, was prepared as a 5 mM 75% DMSO stock and acrylamide as a 400 mM aqueous stock. Okadaic acid was prepared as a 60 μM aqueous stock. RGDS and RFDS peptides were also prepared as aqueous stocks. BSA (Sigma A7906) was Fraction V charcoal and dialysis purified bovine albumin containing 98-99% albumin, with the remainder mostly globulins.

4.2.2 Initiation and maintenance of CDeT27-45(LTu7)-transgenic tobacco calli and suspension cell cultures.

The method used to generate calli and then suspension cell cultures was essentially as described by Hasagawa *et al* (1980). Leaf discs from sterile LTu-7-transgenic young leaves were placed on MS + 30 g l^{-1} sucrose (pH 5.8) with 2,4-D, 0.5 mgl^{-1}, kanamycin 100 mgl^{-1}, agar 1%. Calli were picked as they developed and placed on fresh plates. They were transferred at least twice to fresh plates of this medium every 5 - 7 days. Friable callus was generated by placing the above calli on 30FC medium which consists of 4.3 g l^{-1} MS basal salts mixture, sucrose 30 g l^{-1}, casein hydroslyate 1 g l^{-1}, *myo*-inositol 100 mgl^{-1}, thiamine 0.4 mgl^{-1}, 2,4-D 3 mgl^{-1}, kinetin 0.1 mg l^{-1}, kanamycin 100 mg l^{-1} agar 1%, pH 5.7. The calli were maintained on this medium and transferred every 7 - 10 days. When loose soft callus was repeatedly obtained, a portion was transferred to liquid 30FC medium pH 5. Suspension cultures were maintained in this medium at room temperature with shaking and transferred every 7 - 10 days.

4.2.3 Induction of luciferase expression in suspension cell cultures by osmotic stress.

Suspension cultures used in these experiments were 3 to 7 days old and all the suspension cells used in an experiment were first pooled before distribution. In the experiments reported here, suspension culture cells were aliquoted to give 1-ml settled cell volume in 50-ml Falcon 'Blue cap' polypropylene centrifuge tube containing 5 ml liquid 30FC medium. Each treatment was tested in duplicate and these cultures were incubated with shaking in the laboratory at room temperature.

4.2.4 Luciferase enzyme activity assay

Sufficient suspension culture cells were removed to give approximately 300 μL packed cell volume in an Eppendorf tube. The medium supernatant was removed, the cells were resuspended in 500 μL ice-cold lux buffer (50 mM Na_2HPO_4 pH 7, 50 mM 2-mercaptoethanol, 0.4 M sucrose) and ground with a pestle. The cellular lysate was kept on ice and the cellular debris allowed to settle out. 500 μL cleared cellular lysate was placed in a luminometer cuvette and placed in a LKB Wallac 1251 luminometer. This instrument records light emission as mV from the luciferase enzyme/decanal reaction, which was described in Chapter

2, Materials and Method 2.2.5. Light emission was collected over a 10s period, after a 3s delay to correct for initial autofluorescence. The assays were carried out as described in Materials and Methods 2.2.5. Total soluble protein (TSP) was determined using 5 μL lysate and Bradford (1976) reagent. BSA dilution series was used to generate a protein standard curve. Unless otherwise stated, the data described in each Figure Legend is the average and range in mV/10s/mg TSP observed for duplicate tests of each treatment in a single experiment and the pattern of response was similar between two repeats of each experiment.

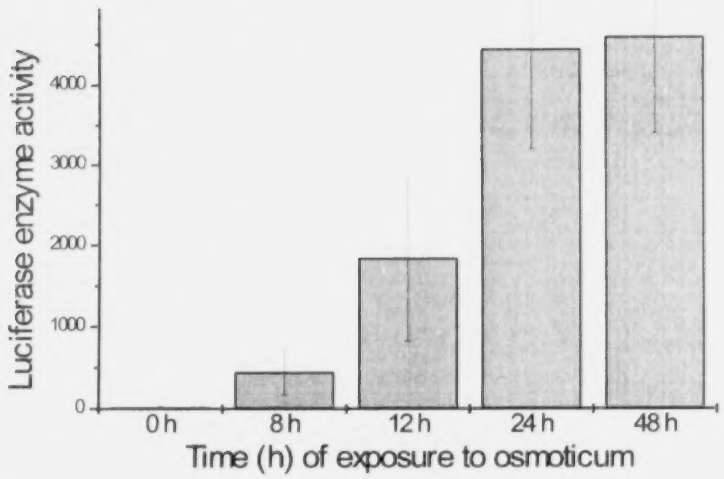
4.3 Results

4.3.1 Luciferase activity is induced by 0.7 M mannitol in a time-dependent manner.

I have previously shown that luciferase activity was inducible in CDet27-45(LTu-7)-transgenic leaf tissue by hyperosmotic stress (Michel *et al* 1993, Chapter 3 Table 3.3.1).

In transgenic cell suspension cultures derived from CDeT27-45(LTu7)-*LuxF* transgenic leaf tissue, the CDeT27-45(LTu7)-*LuxF* gene fusion was highly expressed when switched to medium containing 0.7 M mannitol, i.e. under conditions of osmotic stress (Fig 4.3.1). In the presence of osmoticum and viewed in a microscope, the cells were observed to undergo immediate plasmolysis. The level to which luciferase activity was induced by osmotic stress varied between experiments and probably reflects differences in suspension culture age. Luciferase enzyme activity was detectable by 8 h of osmotic stress application and increased over a 24 h period (Fig 4.3.1).

Figure 4.3.1. Time course of luciferase enzyme induction in response to osmotic stress.

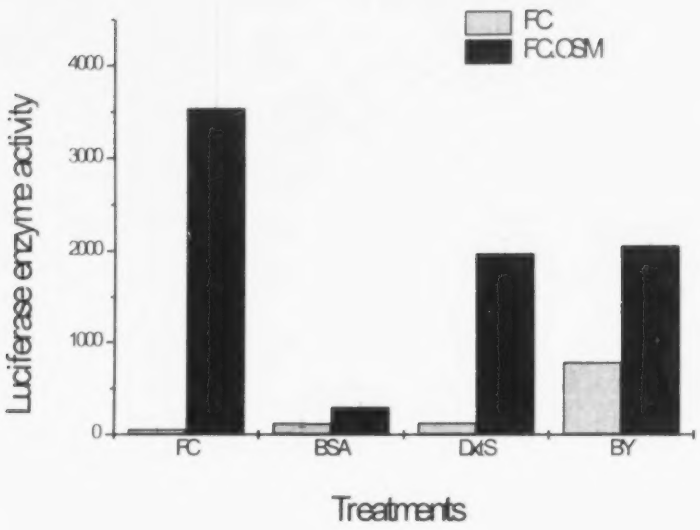


Triplicate suspension cultures were transferred to 30FC liquid medium containing 0.7 M mannitol and incubated as outlined in Materials and Methods (4.2.3). The error bars indicate the standard deviation among the luciferase enzyme activities (mV/10s/mg TSP) expressed by the cell suspension culture samples. This experiment was repeated three times and the same pattern of induction was observed.

4.3.2 Luciferase enzyme activity is also induced by the short-chain fatty acid, butyric acid. The level of luciferase activity induced by osmotic stress is decreased in the presence of 1% BSA.

Luciferase activity was also induced by 10 mM butyric acid, without osmoticum present in the medium. The presence of 1% BSA or 1% dextran sulfate in the medium did not induce luciferase activity, but in the presence of osmoticum, BSA markedly reduced the level of luciferase activity induction. Dextran sulfate did not affect osmotic induction of luciferase activity (Figure 4.3.2).

Figure 4.3.2. Induction of luciferase activity by 0.7 M mannitol, 10 mM butyrate, 1% BSA or 1% dextran sulfate.



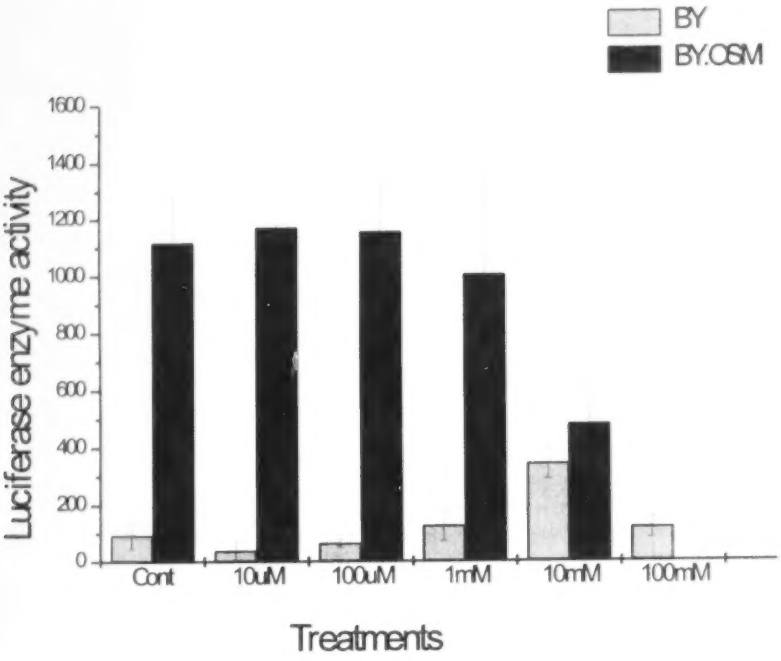
Suspension cultures were aliquoted as outlined in Materials and Methods (4.2.3) and incubated in 30FC liquid medium with (FC.OSM) or without (FC) osmoticum, in the presence of either 10 mM butyrate (BY), 1% BSA (BSA) or 1% dextran sulfate (DxtS) 20 h prior to luminometric analysis of luciferase activity, as outlined in Materials and Methods (4.2.4).

4.3.3 Butyrate induces luciferase activity in suspension cell culture, but not in a dose-dependent manner.

Suspension cultures were incubated in the presence of increasing concentrations of butyrate for 18 h, then analysed for luciferase activity (Figure 4.3.3).

Concentrations of butyrate from 10μM to 1mM did not induce luciferase activity nor affect osmotic induction of luciferase activity. However, 10mM butyrate did induce luciferase activity, although osmotic induction in the presence of 10mM butyrate was reduced. 100mM butyrate did not induce luciferase activity, probably because at such a high concentration there were disruptive effects on cell membranes (Yuli and Oplatka 1987).

Figure 4.3.3. Effect of butyrate on induction of luciferase enzyme activity with and without osmoticum in the medium.



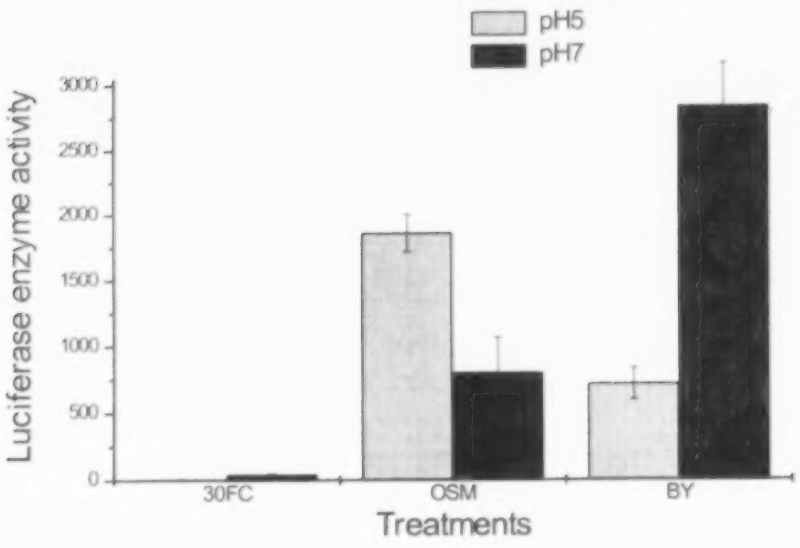
Suspension cells were incubated as outlined in Materials and Methods (4.2.3) in 30FC liquid medium with (BY.OSM) and without (BY) 0.7 M mannitol and containing either 0, 10 μM, 100 μM, 1 mM, 10 mM or 100 mM butyrate diluted from a 1 M aqueous solution. Cells were incubated 18 h before luminometric analysis for luciferase activity as described in Materials and Methods (4.2.4).

4.3.4 The level of butyrate-induction of luciferase activity is pH dependent.

The degree of dissociation of butyric acid into proton and butyrate anion depends upon medium pH. If the inductive effect of butyric acid were due to the "acid loading" and consequent cytoplasmic acidification (the pK_a of butyric acid is 4.8), then at pH 7, fewer protons would be passively carried into the cell and the anion, which is also lipophilic would partition into the cell, but would not cause a decrease in cytoplasmic pH.

This hypothesis was tested by observing the effect of different pH on the induction of luciferase activity. 30FC liquid medium containing 10 mM butyrate, pH 7 was more inductive of luciferase activity than 30FC liquid medium with 10 mM butyrate, pH 5. Osmotically-induced levels of luciferase activity were not increased at neutral pH (Figure 4.3.4).

Figure 4.3.4 Effect of medium pH on osmotic and butyric acid induction of luciferase activity.



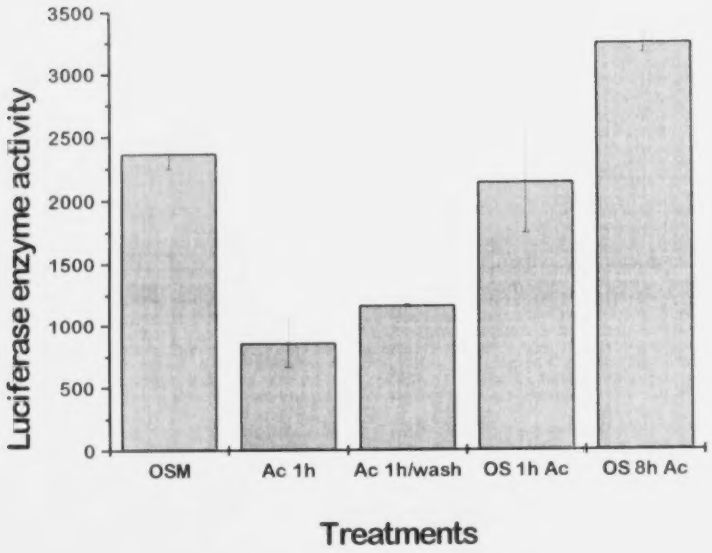
0.7 M mannitol (OSM) or 10 mM Butyrate (BY) was added to 30 FC liquid medium and the medium pH adjusted to either 5 or 7 before filter sterilizing. 30FC liquid medium (Cont) without either osmoticum or butyrate addition, was similarly pH adjusted. Suspension cultures were incubated as outlined in Materials and Methods (4.2.3) for 21 h before luminometric analysis for luciferase enzyme activity as described in Materials and Methods (4.2.4).

4.3.5 Treatment of suspension cells with acrylamide before osmotic induction of luciferase activity results in a decrease in the level of luciferase enzyme activity.

If the cytoskeleton is involved in the osmotic stress signal transduction mechanism, then disruption of the cytoskeletal components involved should affect the cells ability to induce luciferase in response to osmotic stress. Acrylamide (4 mM) has been used in animal cell systems to disrupt intermediate filaments (Wang *et al* 1993) and has been shown to cause nuclear repositioning (Eckert 1885, 1986).

In order to test the effect of acrylamide on osmotic induction, suspension culture cells were treated with 4 mM acrylamide 1 h before application of osmotic stress. This treatment gave rise to a 3-fold reduction in luciferase enzyme activity. Washing the cultures to remove the acrylamide before addition of osmoticum resulted in a similar decrease in luciferase enzyme activity. However treatment of suspension cultures 1 h and 8 h after addition of osmoticum did not affect the levels of luciferase enzyme activity induced by osmotic stress (Figure 4.3.5). This data indicates that acrylamide is not acting as a general metabolic poison but is affecting an early step in the osmotic stress signal transduction pathway.

Figure 4.3.5 Luciferase enzyme activity in suspension cultures treated with 4 mM acrylamide before and after osmotic induction.



Suspension cells were incubated with 4 mM acrylamide (Ac) either 1 h before osmotic stress, with or without (Ac 1h/wash or Ac 1h) washing to remove residual acrylamide, or 1h (OS 1h Ac) or 8 h (OS 8h Ac) after addition of osmoticum. These cultures were incubated in the presence of osmoticum for 18 h before luminometric analysis for luciferase enzyme activity as outlined in Materials and Methods 4.2.4.

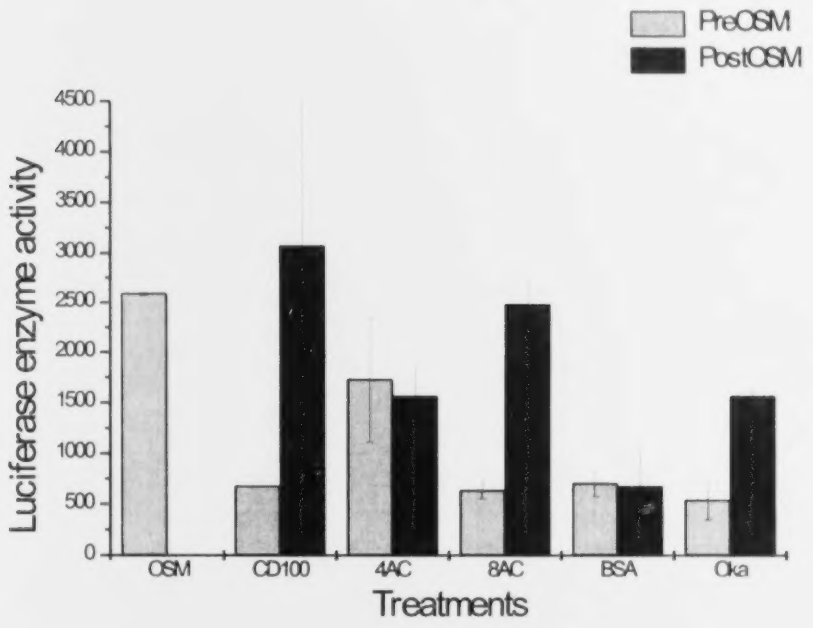
4.3.6 Treatment of suspension cells with cytoskeleton-disrupting compounds before butyrate or osmotic induction, results in a marked reduction of luciferase activity.

Microfilament-disruptive compounds such as cytochalasin D have been used in plant cells to inhibit protoplast spherulation (Abe and Takeda 1989) and cytoplasmic streaming (Cyr 1994 and T Baskin, Univ Missouri, Columbia pers. comm). Okadaic acid is well documented as a phosphatase inhibitor and has also been shown to affect changes in the cytoskeleton, particularly microtubule and intermediate filament integrity (Eriksson *et al* 1992) as phosphatases are essential for maintenance of cytoskeleton integrity. If the cytoskeleton were involved in butyrate- and osmotic- signal transduction, then disruption might affect luciferase activity induction.

Treatment of suspension cells with 100uM cytochalasin D or 8mM acrylamide was found to drastically reduce osmotic induction of luciferase activity (Figure 4.3.6a). The acrylamide concentration was doubled to 8 mM in order to assure sufficient acrylamide to disrupt the cytoskeleton. However, addition after 4 h of osmotic induction did not adversely affect the induction of luciferase activity, which indicates that the effects of acrylamide or cytochalasin D were not generally cytotoxic. Neither

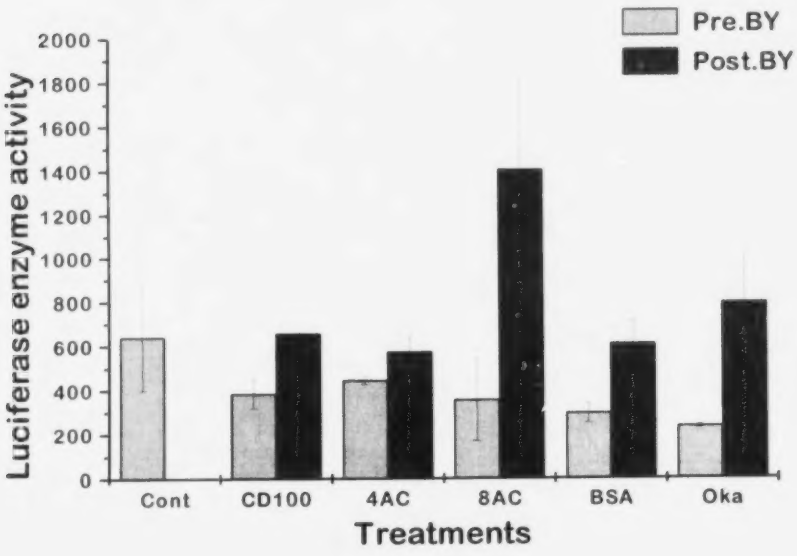
cytochalasin D nor acrylamide reduced butyrate-induction of luciferase activity, and addition of 8 mM acrylamide 4 h after butyrate induction, increased the level of butyrate induction of luciferase activity. The presence of 1% BSA 4 h before or after osmotic induction markedly reduced induction of luciferase activity, while 1% BSA 4 h before, but not 4 h after butyrate-induction, reduced the level of luciferase enzyme activity. Okadaic acid application 4 h before osmotic induction was observed to reduce the level of luciferase enzyme activity induction to 20% of OSM control, while application 4 after induction resulted in a 40% reduction. Treatment with okadaic acid before butyrate induction reduced the level of luciferase enzyme activity, while okadaic acid treatment 4 h after butyrate induction, did not reduced the level of luciferase enzyme activity.

Figure 4.3.6a Effect of cytoskeleton-disrupting compounds cytochalasin D and acrylamide on osmotic induction of luciferase enzyme activity.



Suspension cultures were treated with either cytoskeleton-disrupting compounds cytochalasin D, 100 μM (CD100), 4 or 8 mM acrylamide (4AC, 8Ac) or they were treated with the phosphatase inhibitor okadaic acid, 0.6 μM (Oka) or with 1% BSA for 4 h before (Pre-OSM) induction of luciferase activity with 0.7 M mannitol or 4 h after (Post-OSM) osmotic induction of luciferase activity. Cells treated with just 0.7 mannitol were included as a control (OSM). All cell samples were incubated for 24 h in the presence of osmoticum before luminometric analysis for luciferase enzyme activity.

Figure 4.3.6b Effect of cytoskeleton-disrupting compounds cytochalasin D and acrylamide on butyric acid induction of luciferase enzyme activity.



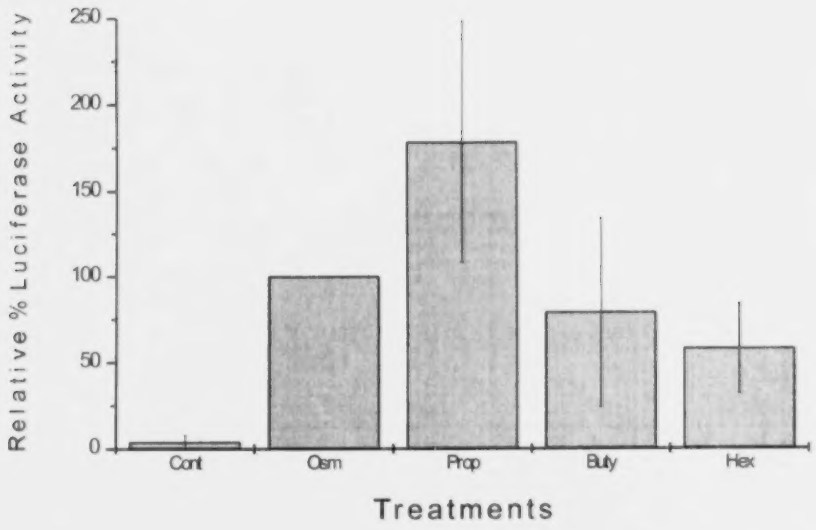
Suspension cultures were treated with either cytoskeleton-disrupting compounds cytochalasin D, 100 μM (CD100), 4 mM or 8 mM acrylamide (4AC, 8AC) or they were treated with the phosphatase inhibitor okadaic acid, 0.6 μM (Oka) or with 1% BSA for 4 h before (Pre-BY) induction of luciferase activity with 10 mM butyric acid or 4 h after (Post-BY) butyrate induction of luciferase activity. Cells which were not treated or were treated with 10 mM butyrate were included as controls (Cont). All cell samples were incubated for 24 h with butyrate before luminometric analysis for luciferase enzyme activity.

4.3.7 Other short-chain fatty acids in the homologous series propionate to hexanoic acid induce luciferase activity.

SCFAs such as propionate and butyrate have been shown to decrease cytosolic pH by "acid loading" (Frachisse *et al* 1988). The pK_a of the homologous series acetate to hexanoic acid is approximately 4.8 so that once the SCFA molecule has diffused across the plasma membrane it would tend to be in its dissociated form in the neutral pH of the cytoplasm (Reid *et al* 1989), thus releasing protons.

Treatment of suspension cells with 10 mM propionate, butyrate or hexanoic acid at pH 7 resulted in induction of luciferase activity (Fig 4.3.7).

Figure 4.3.7 Induction of luciferase enzyme activity by short-chain fatty acids.



Suspension cultures were incubated for 15 h with 30FC liquid medium containing either 10 mM propionic acid (Prop), butyric acid (Buty) or hexanoic acid (Hex). The pH of these media were adjusted to 7, then filter sterilized before addition of the cells. Suspension cells in 30FC liquid medium with (Osm) and without (Cont) 0.7 M mannitol were included as a check on luciferase enzyme inducibility in these cultures. The data is shown as the average Relative % luciferase activity in two independent experiments, with the luciferase activity (as mV/10s/mg TSP) generated by cells in Osm taken as 100%. The bars indicate the range of the Relative % luciferase enzyme activity in these two experiments.

4.3.8 Treatment of cell suspension cultures with K^+-ionophore valinomycin, H^+-ATPase stimulator fusicoccin, did not eliminate osmotic induction of luciferase activity.

Changes in membrane polarization as well as changes in cytoplasmic pH have been shown to occur concomitantly with cell growth (Kropf and Gibbon 1989) as well as with certain environmental changes, such as light (Felle and Bertl 1986), external osmotic potential (Reinhold *et al* 1984, Reuveni *et al* 1987) and application of phytohormones such as auxin (Felle *et al* 1986) and abscisic acid (Gehring *et al* 1990). If membrane polarization or pH changes were involved in the osmotic stress signal transduction pathway which culminates in the induction of luciferase enzyme activity, then treatment of CDeT27-45(LTu-7) suspension cultures with compounds which have been shown to affect membrane polarization and/or pH should affect the osmotic induction of luciferase activity.

Suspension cultures were incubated with either 10μM valinomycin which has been shown to perturb membrane polarization by carrying potassium ions into plant cells (Glaser and Donath 1989), 10μM fusicoccin which is reported to cause membrane polarization changes via

stimulation of H^+-ATPase (Marré 1979, Johansson *et al* 1993), 10 μM ruthenium red which inhibits Ca^{++} ion release from internal stores (Subbaiah *et al* 1994) or 10 μM FCCP, a proton carrier (Poole *et al* 1984).

Table 4.3.1 is a composite of three experiments conducted to test the effects of compounds described above which affect membrane depolarization status or the ability of membranes to release calcium ions from internal stores. The data is presented as luciferase activity relative to that obtained from osmoticum alone (which was taken as 100% for each experiment).

Osmotic induction of luciferase activity was not significantly reduced by either fusicoccin or valinomycin treatment but treatment with ruthenium red markedly reduced luciferase enzyme activity. However, induction was not restored in the ruthenium red treated cells by concomitant treatment with calcium. FCCP treatment drastically reduced luciferase induction (Table 4.3.1).

Table 4.3.1 Effect of membrane depolarizing compounds on osmotic induction of luciferase activity

Treatment	Relative % induction of luciferase activity	
Osmoticum	100	
+ fusicoccin	70	+/- 16
+ valinomycin	100	+/- 37
+ ruthenium red	32	+/- 16
+ ruthenium red +Ca	38	+/- 11
+ FCCP	12	+/- 1
Mas-luxF+auxin+FCCP	3	
Mas-LuxF+auxin	500	

Suspension cells were incubated in 30FC medium containing 10 μM fusicoccin diluted from a 10mM DMSO stock, 10 μM valinomycin diluted from a 10mM DMSO stock + 5 mM KCl, 10 μM ruthenium red diluted from a 400 mM aqueous stock +/- 5mM $CaCl_2$ or 10 μM FCCP diluted from a 10mM DMSO stock, for 30 min prior to addition of osmoticum. The above data represented the average and standard deviation among three independent experiments in which the data was expressed as % relative to osmoticum treatment alone which was taken as 100% with the exception that *Mas*-LuxF + auxin +/- FCCP data was from one experiment .

The effect of FCCP was probably a general metabolic one. This was shown by suspension cells transformed with an auxin-activated promoter (*mas*-LuxF), described in Chapter 2, Materials and Methods 2.2.4, which were similarly treated with FCCP. While increased auxin stimulated the *mas*-promoter, in the presence of auxin + FCCP, luciferase activity was eliminated. FCCP is a potent uncoupler of oxidative phosphorylation and as such would completely

disrupt the energy balance of these cells. As gene transcription and translation is an energy-requiring process which would be adversely affected by oxidative phosphorylation uncoupling, a direct effect by FCCP on luciferase induction mechanism cannot be discerned from these experiments. Treatment with ruthenium red also significantly reduced luciferase induction, but concurrent treatment with elevated calcium did not rescue induction of luciferase activity which suggests that ruthenium red had other effects.

4.4 Discussion

Hyperosmotic stress as well as 10mM butyrate induces luciferase enzyme activity in CDet27-45*LTu7* transgenic suspension culture cells. The presence of 1% BSA in the incubation medium reduced both osmotic- and butyrate-induction of luciferase enzyme activity. BSA has been shown to bind free fatty acids (Earnshaw and Truelove 1970) and to also bind to plasma membrane as a non-specific ligand (Wang *et al* 1993). BSA has been shown to bind 3 to 4 moles of fatty acid per mole of BSA (Chen 1967). 1% BSA (approximately 0.1mM) would be expected to bind 0.3-0.4mM butyrate and therefore elimination of luciferase activity induction by BSA was unlikely to be the result of binding to short chain fatty acids and reducing the concentration below the required threshold value. BSA may be stabilizing the membrane or the osmotically-sensitive receptor so that it cannot respond to either osmotic or butyrate induction of luciferase activity. BSA has been shown to penetrate plant cell walls but it does not penetrate the plasma membrane (Baron-Epel *et al* 1988). The elimination of osmotic-induction of luciferase activity in the presence of BSA suggests that BSA is binding to a mechano-sensitive receptor. However dextran sulfate, a molecular crowding and protein stabilizing compound, did not induce

luciferase activity nor did it reduce osmotic induction of luciferase activity, which suggests a direct effect of BSA on luciferase activity induction.

Butyrate-induced luciferase activity required 10mM butyrate before marked induction was observed. That such a high concentration was necessary before luciferase enzyme activity was induced suggests that the butyrate effect was the result of structural change to some component, such as proteins or membranes, involved in induction pathway (Figure 4.3.3). Considering that the level of induction by propionate or butyrate was increased at neutral pH and that at neutral pH, neither propionate nor butyrate would decrease cytoplasmic pH by acid-loading as they would in their protonated form, the effect of these SCFAs may be via membrane perturbation. Propionate at pH 7 has been shown to increase membrane fluidity (Yuli and Oplatka 1987) and therefore may affect membrane/protein or protein/protein interactions. These results suggest that cytosolic pH acidification was not the initial signalling event, but that a membrane-dependent event was the initial step in this signal transduction pathway.

It has been suggested that the perception of osmotic stress is mechano-sensitive and involves the cytoskeleton

(Davies 1993). Cytoskeleton-disrupting compounds such as cytochalasin D have been shown to affect plant cell processes such as protoplast fusion and cytoplasmic streaming (Abe and Takeda 1989), which require an intact cytoskeleton. Pre-treatment of suspension cells with cytochalasin D markedly reduced the induction of luciferase activity by osmoticum, yet similar treatment, after induction had occurred, did not eliminate the response of induced luciferase activity. This suggests that the cytoskeleton was involved in the osmotic signal transduction mechanism. Treatment of the suspension cells with okadaic acid which is a potent phosphatase inhibitor in plants, also reduced the osmotic and butyrate induction of luciferase activity. Phosphatases are required for cytoskeletal integrity since phosphorylation of microfilaments results in cytoskeleton disintegration (Eriksson *et al* 1992). Okadaic acid may shift the cytoskeleton dynamics towards increased phosphorylation and as such could compromise the ability of the cytoskeleton to transduce the signal. However, okadaic acid could also be affecting the phosphorylation state of other required protein(s) in the signal transduction pathway.

While there is no direct evidence for the existence of plant cell intermediate filaments, the presence of

intermediate filaments has been suggested by selective extraction and keratin (a type II IF of animal cells) antibody binding (Yang *et al* 1992). Certainly acrylamide has been shown in animal cells to affect IF integrity and to cause a shift in nuclear position (Wang *et al* 1993, Eckert 1986). Acrylamide also eliminated osmotic-induction of luciferase activity (Figure 4.3.6a). Treating osmotically-induced cells 1 h after osmotic induction, however, did not reduce osmotic induction of luciferase activity (Fig 4.3.5), so it is unlikely that the effect of acrylamide is a general cytotoxic one. However, acrylamide treatment did not reduce butyrate-induction of luciferase activity (Figure 4.3.6b) which suggests that disruption or lack of tension in the cytoskeleton alone is insufficient to induce luciferase enzyme activity. Interestingly, 8 mM acrylamide added 4 h after butyrate-induction more than doubled the level of luciferase enzyme activity. As treatment of plant cells with butyrate has been observed to cause a decrease in actin tension within the cytoskeleton (M Schindler, Michigan State university, pers comm), this suggests that the effect of butyrate and acrylamide are cumulative. However, a decrease in cytoskeletal tension is unlikely to be the only step in the signal transduction pathway as neither cytochalasin D nor acrylamide treatments increase osmotic induction of luciferase enzyme activity. This

data suggests that butyrate must affect another component of the signal transduction pathway as well.

As outlined in the Introduction, section 1.12, the effect of butyrate on histone acetylation and consequent chromosome decondensation in plant cells has not been clearly established. Possibly the induction of luciferase enzyme activity by butyrate was the result of increased transcription due to chromosome decondensation. However, this seems unlikely as the presence of BSA eliminated both osmotic- and butyrate-induction of luciferase activity, yet butyrate concentration would not be markedly reduced in the presence of 1% BSA (Chen 1967).

The observation that neither fusicoccin nor valinomycin induced or inhibited osmotic-induction of luciferase activity (Table 4.3.1) suggested that membrane polarization changes were not involved in this signal transduction pathway. The negative effect of ruthenium red on osmotic induction of luciferase enzyme activity could not be reversed by elevated calcium which contrasts with the results obtained by Subbaiah *et al* (1994) investigating anoxic induction of maize *adh1* gene. This suggests that the effect of ruthenium red in tobacco cells was more pleiotropic and that calcium effects did not seem to be involved directly in osmotic induction of

luciferase activity.

Other short-chain fatty acids also induce luciferase activity in suspension cells cultured in 30FC medium pH 7 (Figure 4.3.7). This supports the model that these short-chain fatty acid molecules and not cytoplasmic acidification *per se* affected the induction of luciferase activity. The observation (Figure 4.3.7) that hexanoic acid, a six carbon SCFA induced luciferase enzyme activity to a lesser extent than propionic acid, a three carbon SCFA, may be due to the deleterious effects that longer short-chain fatty acids are known to have on mitochondria (Earnshaw and Truelove 1970) and does not represent a real difference in induction of luciferase activity among the short-chain fatty acids. This data supports the model that perturbation of the plasma membrane, either by plasmolysis resulting from hyper-osmotic stress or treatment with lipid soluble short-chain fatty acids, results in induction of luciferase enzyme activity.

Taken together, these data suggest that the plasma membrane and cytoskeleton are involved in the signal transduction mechanism.

5 The induction of luciferase activity in CDeT27-45(LTu-7) transgenic tobacco suspension cultures in response to osmotic stress is dependent upon protein receptor/linkers external to the plasma membrane.

5.1 Introduction

The mechanisms by which plant cells sense changes in water status or cell turgor are unknown. It has been suggested that osmotic stress is perceived by mechanosensitive receptors/linkers present on the plasma membrane-cell wall interface (Davies 1993, Pont-Lezica *et al* 1993, Wyatt and Carpita 1993, Schindler *et al* 1989). Evidence linking the cell wall, plasma membrane and cytoskeleton as important determinants of plant development is beginning to accumulate. Katsuta and Shibaoka (1988) have presented data which shows that inhibiting cell wall regeneration in protoplasts affects nuclear positioning. Similarly, actin filament disruption also inhibits nuclear repositioning. In the fertilized eggs of *Fucus*, the formation and fixation of the developmental axis are dependent on the polarized organization of the microfilaments (Brawley and Robinson 1985). Berger *et al* (1994) have shown that the cell wall is also necessary for maintenance of the rhizoid-thallus

differentiated state and to direct cell fate in *Fucus* embryo development.

In cultured tobacco cells, Akashi *et al* (1990) reported that cortical microtubules must maintain an association with the cell wall if they are to maintain their stability at low temperatures. Akashi and Shibaoka (1991) suggested that the mechanism of interaction between extracelluar signals and cortical cytoskeleton is associated with some components of the plasma membrane. They observed a loss of microtubule fragments on membrane ghosts prepared from protoplasts pre-treated with protease or trypsin. In addition, they found that exogenously applied extensin, a family of hydroxyproline-rich glycoproteins, or poly-L-lysine increased cold-stability of cortical microtubules in control protoplasts, but not in protease-pretreated protoplasts. Masuda *et al* (1991) observed both proteolytic enzyme digestion and hypertonic stress induced abnormal cytoplasmic streaming in the mesophyll cells of the aquatic angiosperm *Vallisneria*, which suggested that an extracellular receptor was linked to the cytoskeleton.

If the mechanism by which plant cells perceive osmotic stress involves a receptor/linker, then proteolytic digestion by cell wall degrading enzymes could eliminate

the osmotic induction of luciferase activity.

An alternate hypothesis concerning the mechanism of osmotic induction of luciferase activity is that cell wall fragments or lipid membrane components released upon plasmolysis may act as inducers of luciferase activity, similar to the mechanism by which cell wall fragments released by pathogenic microbes act as elicitors and trigger specific stress responses in plant cells (Lamb *et al* 1989, Côté and Hahn 1994, Introduction 1.2.2).

In this chapter, I outline the effects of limited digestion with enzymes which hydrolyse specific cell wall components, on osmotic- and butyrate-induction of luciferase enzyme activity.

5.2 Materials and Methods

5.2.1 Materials

The enzymes and chemicals used in these experiments were obtained from Sigma Chemical Company, St Louis, Missouri, USA with the exception of driselase which was obtained from Fluka Biochemicals. Enzyme concentrations specific to each experiment are given in Results figure legends or text. The Y23 enzyme (Ishii 1976) used in these

experiments was an endopolygalacturonase mix of polygalacturonase, pectin lyase and maceration-stimulation factor and was resuspended in 30FC liquid medium at 0.01% (w/v), then filter sterilized. Based upon published Y23-polygalacturonase activity levels, 0.01% contains 4 units of polygalacturonase activity (Nagata and Ishii 1979).

5.2.2 Initiation and maintenance of CDeT27-45 transformed tobacco callus and suspension cell cultures.

Suspension cell cultures were initiated from callus tissue generated from CDeT27-45(Lux-7) transgenic leaves as outlined in Chapter 4.2.2. Cell cultures were maintained as friable callus and as suspension cultures as outlined previously (Materials and Methods 4.2.3). Suspension cells used in each experiment were first pooled and then dispensed as duplicate samples for each condition tested.

5.2.3 Induction of luciferase expression in suspension cell cultures by osmotic stress and luciferase enzyme assay by luminometry.

Induction of luciferase activity in suspension cell cultures was as outlined previously in Chapter 4.2.3.

Luciferase assays were carried out using an LKB-Wallac 1251 luminometer. The procedure was the same as outlined in Chapter 4.2.4. Unless otherwise stated, the data shown in Results was the average mV/10s/mg TSP luciferase enzyme activity observed for the duplicate tests of each treatment and the lines represent the range of values obtained. The pattern of response was similar between two repeats of each experiment.

5.2.4 Method used to check cell-wall digesting enzymes for contaminating proteases.

The method used to test the cell-wall digesting enzymes for contaminating proteases was essentially as described by Baldwin (1978). Each assay consisted of 10μl luciferase enzyme from a 1 mg/ml aqueous stock + 100 μl enzyme solution, containing 4 units of one of the enzymes listed in Table 5.3.1. Triplicate assays were incubated at room temperature in a total volume of 1 ml Lux- Buffer (50 mM Na_2HPO_4 pH 7, 50mM β-mercaptoethanol, 0.4M sucrose). 5μl samples were withdrawn from each assay, immediately after luciferase addition as time-zero and at 1 h and 7 h and placed in 1ml lux- buffer for luminometric analysis as described in Materials and Methods 4.2.4

5.3 Results

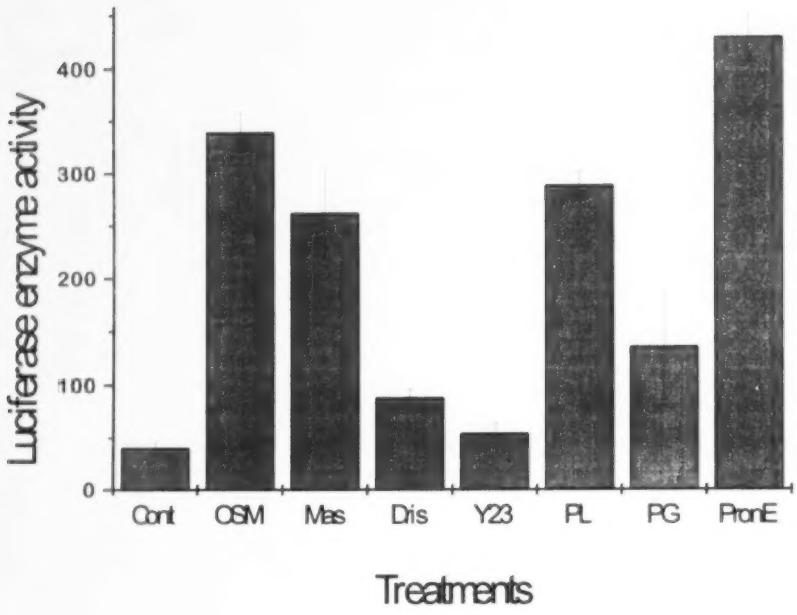
5.3.1 Partial cell wall digests result in varying losses of luciferase induction, and were dependent upon the cell wall digesting enzymes used.

If cell-wall-plasma-membrane linkers or osmotically-sensitive receptors play a role in the osmotic-stress signal transduction mechanism, and are located externally or partially externally to the plasma membrane, then limited digestion with appropriate cell wall degrading enzymes or proteinases should affect the osmotic induction of luciferase activity.

To test this hypothesis, suspension cells were treated with 4 units of cell-wall digesting enzymes for 30 min before immersion in osmoticum. Inspection of enzyme-digested cell suspension cultures through a microscope showed that cells had not lost their cell walls and protoplasts were not generated from these cultures. Their cell walls appeared as intact as undigested cultures which suggests that the time of digestion and/or the limited concentration of enzyme used in these experiments was insufficient to fully remove the cell wall.

Data presented in Figure 5.3.1 show that cellulase (macerase), purified pectin lyase (PL), and a protease (pronase E) did not markedly reduce osmotic induction of luciferase activity. However, pre-treatment of cells with driselase and Y23, both of which contain endopolygalacturonase and pectin lyase, and polygalacturonase (PG), reduced the induction of luciferase activity in response to osmotic stress.

Figure 5.3.1. Effect of cell-wall digesting enzymes on the osmotic induction of luciferase enzyme activity.



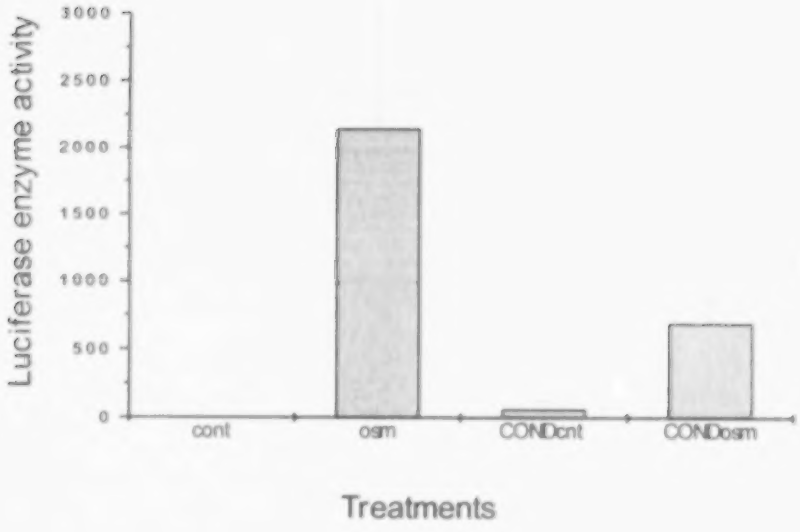
Suspension cells were treated with 4 units of either mascerase (Mas), driselase (Dris), endopolygalacturonase enzyme mixture (Y23), purified pectin lyase (PL), purified polygalacturonase (PG) or pronase E (PronE) for 30 min, centrifuged and resuspended in 30FC liquid medium + 0.7 M mannitol for 15 h before luminometric analysis for luciferase activity. Undigested cells were suspended in 30FC liquid medium + 0.7 M mannitol (OSM) and 30FC liquid medium (Cont) as controls. The data shown was the average mV/10s/mg TSP observed for duplicate tests of each treatment and the lines represent the range of values obtained. The pattern of response was similar among three repeats of this experiment.

5.3.2 Osmotic induction of luciferase activity was decreased in conditioned medium.

The hypothesis that a receptor is involved in sensing osmotic stress necessitates the existence of a ligand, which upon binding to the receptor, instigates a signal transduction mechanism. Work on plant perception of pathogen attack has shown that cell wall fragments can act as signalling molecules that bind to specific receptors (Basse and Boller 1992). To ascertain whether endopolygalacturonase enzymes (Y23, Driselase or PG) themselves or cell wall fragments generated by the polygalacturase enzyme activity were activating the signal transduction mechanism which resulted in the induction of luciferase enzyme activity, suspension cells were pre-treated with conditioned medium. Conditioned medium was generated by digesting suspension culture in 30FC liquid medium or 30FC liquid medium + 0.7 M mannitol, then pelleting the cells. This medium was heat denatured to remove enzyme activity, but contained the cleavage products of endopolygalacturonase.

The data shown in Figure 5.3.2 indicate that endopolygalacturonase cleavage products did not induce luciferase enzyme activity but they did markedly reduce the osmotic induction of luciferase activity.

Figure 5.3.2. Osmotic induction of luciferase activity in cell suspension cultures which were pre-treated with conditioned medium.

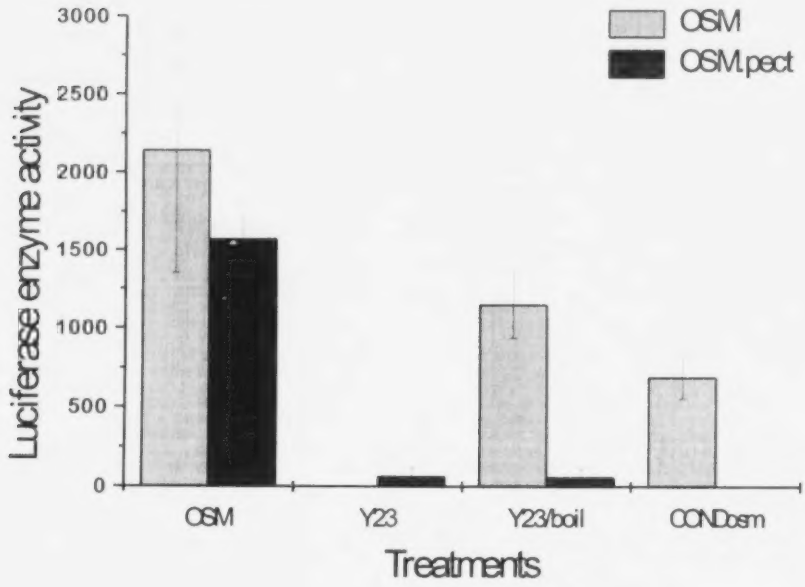


Conditioned medium was 30FC liquid medium with or without 0.7 M mannitol in which suspension cells had been digested with 0.01% Y23 for 60 min, then the medium supernatant boiled 20 min to denature Y23. Suspension cells were incubated with an equal volume of conditioned medium for 30 min before addition of either fresh 30FC liquid medium (CONDcnt) or 30FC + 0.7 M mannitol (CONDosm). Duplicate samples which were not pretreated with conditioned medium before addition of 30FC liquid medium (Cont) or 30FC + 0.7 M mannitol (OSM) were included as controls. The cultures were incubated as outlined in Materials and Methods 4.2.3 for 18 h before luminometric assay for luciferase activity as described in Materials and Methods 4.2.4.

5.3.3 Y23-digestion products affect osmotic induction of luciferase activity.

In order to test whether Y23 enzyme proteins, either denatured or native, were affecting osmotic induction of luciferase activity, Y23 which had been denatured by boiling water-bath treatment, 1% pectin digested with Y23, as well as conditioned medium were used to pre-treat suspension cell cultures. The data shown in Figure 5.3.3. indicate that pectin fragments generated by Y23 digestion are suppressing osmotic induction of luciferase enzyme activity as denatured Y23-digested pectin markedly reduced osmotic induction while denatured Y23 enzyme alone, did not. Pre-treatment of suspension cells with conditioned medium which would contain pectic wall fragments, also reduced osmotic induction of luciferase enzyme activity.

Figure 5.3.3. Effect of Y23 and Y23 digestion-products on osmotic induction of luciferase activity.



Suspension cells were pre-treated for 30 min with 0.01% endopolygalacturonase enzyme (Y23) with or without 1% pectin, denatured 0.01% Y23 either without 1% pectin or with 1% pectin, but which was denatured after 30 min pectin-digestion. Suspension cells were also pre-treated with conditioned medium as outlined in Figure 5.3.2 or were treated with osmoticum with or without pectin (OSM) as a control. The cultures were incubated in osmoticum 18 h as outlined in Materials and Methods 4.2.3 before luminometric analysis for luciferase activity as described in Materials and Methods 4.2.4.

To check that the cell wall degrading enzymes used in these experiments, namely Y23, purified PG and purified PL, did not contain any residual protease activity, these enzymes were individually incubated in the presence of purified luciferase enzyme and the activity of the luciferase enzyme was monitored at 1 h and 7 h (Table 5.1). Luciferase enzyme activity was not reduced by these enzymes over 7 h of incubation. Reduction in enzyme activity in the untreated luciferase sample after 7 hr, indicates that enzyme function decreased over the incubation period, but protease activity was not contributing to the reduction in luciferase enzyme activity in samples pre-treated with these enzymes.

Table 5.3.1 Luciferase assay for protease activity.

time:	0	1 h	7 h
	luciferase activity :	mV / 10s / mg TSP	
enzyme			
none	464 +/- 130	418 +/- 15	324 +/- 190
Y23		554 +/- 23	300 +/- 4
PG		572 +/-62	554 +/- 74
PL		521 +/-7	492 +/- 55
pronaseE		16 +/-2	
trypsin		18 +/-7	
protease		13 +/- 1	

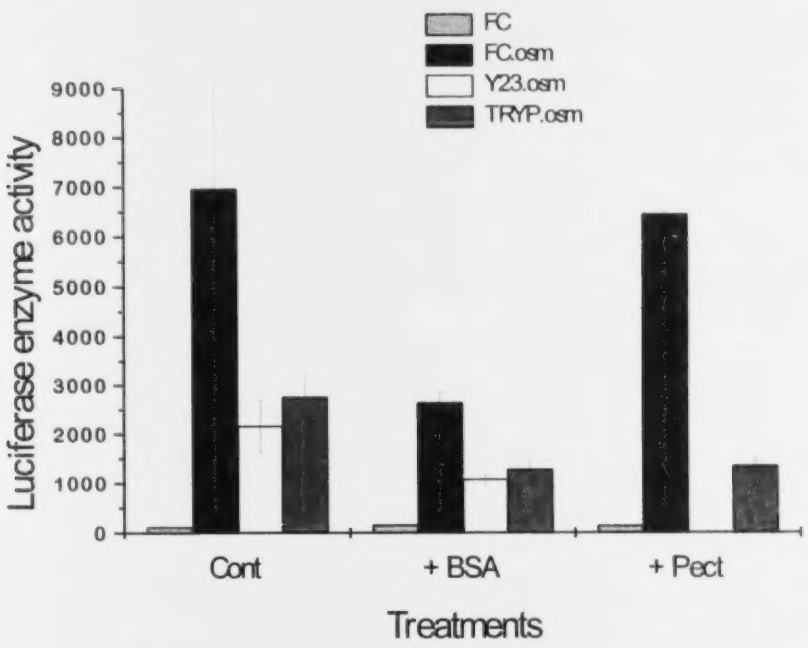
The protocol used to assess the protease contamination in the cell-wall digesting enzymes listed above is described in Materials and Methods 5.2.4. The data shown indicates the average luciferase enzyme activity and the standard deviation among the triplicate assays at each time point.

The results shown in Figures 5.3.2 and 5.3.3 indicate that while digestion products from Y23 do not induce luciferase activity, they do suppress the osmotic induction of luciferase activity. As the endopolygalacturonase enzymes used did not display any protease activity, the suppression of osmotic induction of luciferase enzyme activity by treatment of suspension cell cultures with Y23-conditioned medium or Y23-digested pectin, indicates that pectic fragments generated by endopolygalacturonase digestion are affecting the osmotic stress signal transduction pathway. This suggests that pectic fragments may be binding a receptor so that the osmotic stress signal transduction mechanism was eliminated.

5.3.4 Effect of trypsin digestion, BSA and pectin on osmotic induction of luciferase activity.

The signal transduction mechanism was found to be affected by proteolytic digestion with trypsin (Figure 5.3.4). In order to test whether an alternate protein substrate would alter the effect of proteolytic digestion, BSA (1%) was included in some samples as a protein source.

Figure 5.3.4. The effect of 1% BSA and 1% pectin on osmotic induction of luciferase activity with and without enzyme pre-digestion.



Suspension cells were digested with 0.01% of either endopolygalacturonase (Y23 - white histogram) or with trpysin (TRYP - dark grey) in 30FC liquid medium alone or containing 1% BSA (+BSA) or 1% pectin (+Pect). Cells were digested for 30 min, then mannitol solution was added to give 0.7 M to all samples except FC (light grey histogram). Suspension cells were also treated with 30FC liquid medium + 0.7 M mannitol (osm - black histogram) alone or with 1% BSA (+BSA) or 1% pectin (+Pect). The suspension cells were incubated for 18 h before assaying for luciferase activity.

Both Figures 5.3.3.and 5.3.4 show that 1% pectin alone did not induce luciferase activity nor did pectin suppress osmotic induction of luciferase activity. 1% BSA, however, did reduce the level of luciferase activity induced by osmotic stress (Fig 5.3.4). Treatment of cells with Y23 reduced the osmotic induction of luciferase activity and completely eliminated any induction of luciferase enzyme activity if 1% pectin was included in the digestion mix (Figures 5.3.4).

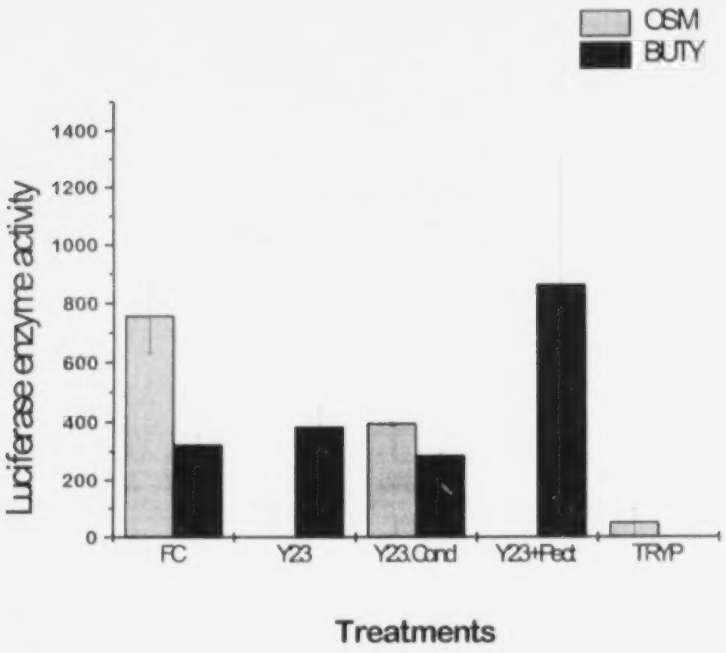
5.3.5 Trypsin digestion eliminates osmotic and butyrate induction of luciferase activity.

To test whether trypsin, a substrate-specific protease which cleaves preferentially lysine and arginine peptide bonds, would reduce butyrate induction of luciferase activity similar to the reduction of osmotically-induced luciferase activity (Figure 5.3.4), suspension cells were treated as outlined in Figure 5.3.5. Treatment with trypsin markedly reduced both osmotic and butyrate induction of luciferase activity. The presence of 1% BSA with trypsin further decreased the osmotic induction of luciferase activity. As osmoticum + 1% BSA also showed reduced induction of luciferase enzyme activity, it is unlikely that a peptide cleavage product was responsible for this observed inhibition/suppression. The presence of

1% BSA results in a further reduction in the osmotic induction of luciferase activity in trypsin-digested suspension cells which suggests that a BSA-specific effect was responsible for the decrease in osmotic induction of luciferase activity in the Y23 + BSA and trypsin + BSA treated samples.

Data presented in Figure 5.3.5. indicate that while digestion of suspension cells with Y23 and trypsin essentially eliminated the osmotic induction of luciferase enzyme activity, only trypsin-digestion eliminated butyrate- and osmotic-induction of luciferase enzyme activity. Digestion of suspension cells with Y23 did not affect subsequent induction of luciferase activity by 10 mM butyrate. Even the presence of Y23 digested pectin (Y23.Cond and Y23+Pect) did not suppress butyrate induction of luciferase enzyme activity, which suggests that butyrate induction does not involve ligand-receptor binding. However, the reduction in osmotically - induced luciferase activity suggests that a component of the osmotically-sensitive signal transduction system which is sensitive to BSA and Y23 digestion products, must be external or partially external on the plasma membrane. Such a component might be a receptor which is bound or stabilized by BSA and suppressed by Y23-digestion products.

Figure 5.3.5 The effect of Y23, trypsin and conditioned medium on osmotic and butyrate induction of luciferase enzyme activity.

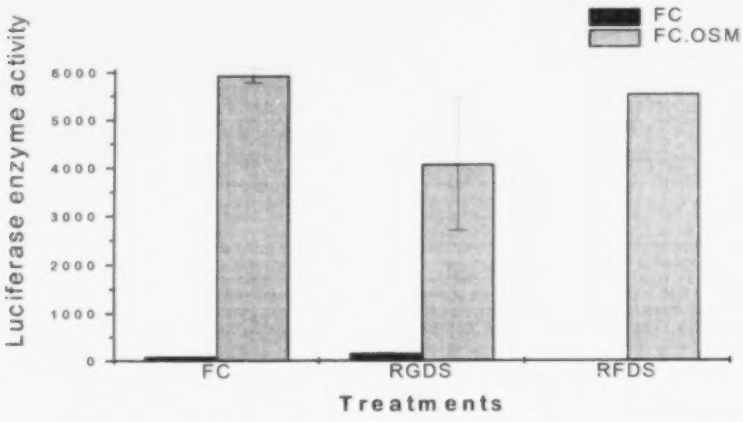


Suspension culture was treated with 0.01% Y23 or trypsin in 30FC liquid medium for 1 h, centrifuged and the supernatant removed as conditioned medium. Fresh suspension culture was then incubated with 0.01% enzyme or an equal volume of conditioned medium for 30 min before addition of 0.7 M mannitol (grey histographs) or butyrate to give 10 mM final concentration (Black histographs). Suspension cultures were also left untreated with either enzyme or conditioned medium (FC) but were subjected to osmoticum or butyrate as controls.

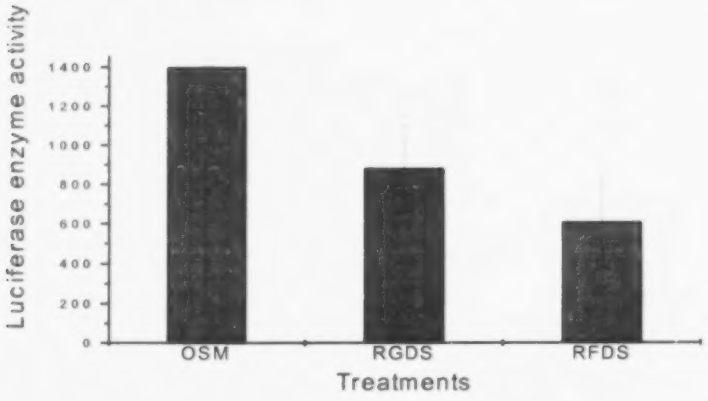
5.3.6 RGDS peptides do not specifically reduce osmotic induction of luciferase activity.

The integrin-binding peptide sequence arginine-glycine-aspartate-serine (RGDS at 500 μM) has been shown to affect cytoplasmic streaming in *Chara* cells in response to gravity, while RFDS peptide (glycine residue substituted with phenylalanine) did not (Wayne *et al* 1992). In order to test if integrin-binding sequence RGD had an effect on the osmotic signal transduction pathway, suspension culture cells were incubated with 500 μM RGDS peptide or with 470 μM RFDS peptide as a control for 4 h (Figure 5.6a) or for 24 (Figure 5.6b) before application of osmoticum.

Figure 5.6 The effect of RGDS peptide on osmotic induction of luciferase enzyme activity.



a Suspension cells were pretreated for 4 h with 500μM RGDS or 470μM RFDS before osmoticum (FC.OSM) was added and then were incubated a further 24 h before luminometric analysis for luciferase enzyme activity.



b Suspension cells were pretreated as in **a** except that they were incubated with the peptides for 24 h before addition of osmoticum. These suspension cells were incubated in osmoticum for 15 h before luminometric analysis for luciferase activity. Data in **a** and **b** show the mV/10s/mg TSP observed for duplicate tests of each treatment in one experiment and the bars indicate the range of values obtained. The pattern of response was similar between two repeats of this experiment.

RGDS did not induce luciferase activity nor did it markedly reduce osmotic induction of luciferase activity (Figure 5.3.6a). RFDS peptide which has phenylalanine instead of glycine, was used as a control. Both RGDS peptide and RFDS peptide control reduced osmotic induction of luciferase activity (Figure 5.6b) when cells were incubated with these peptides for 24 h, which may indicate a non-specific effect such as a general stabilization of cell wall-plasma membrane architecture.

5.4 Discussion

The initial observation that treatment of suspension cell cultures with cell wall degrading enzymes resulted in the loss of osmotic induction of luciferase activity suggested that an osmotically sensitive receptor might be cleaved, which resulted in the loss of induction. Data shown in Figure 5.3.2 showed that hydrolysed cell wall fragments do not induce luciferase activity, and data shown in Figure 5.3.3 indicated that conditioned medium which would contain pectic cell wall fragments, suppresses osmotic induction of luciferase enzyme activity. These data suggest that pectin fragments may bind to an osmotically-sensitive receptor without eliciting the signal transduction mechanism, whereas these fragments do prevent the osmotic induction of luciferase activity. This is similar to the suppression of the ethylene-induction stress response in tomato by oligosaccharides released from tomato cell walls by pathogenic fungi (Basse and Boller 1992).

At the particular concentration and pre-digestion time used in these experiments, proteases such as pronase E, a microbial acid protease which is isolated from *Streptomyces griseus* and whose specificity is directed towards the size of the peptide substrate (Sodek and

Hofmann 1970), did not reduce the osmotic induction of luciferase activity (Figure 5.3.1). However, pre-digestion with trypsin markedly reduced osmotic induction (Figure 5.3.4). Trypsin, isolated from bovine pancreas, is a serine protease which preferentially cleaves lysyl-arginyl residues (Walsh and Wilcox 1970). These differences in protease specificity may explain their different effects on osmotic induction of luciferase activity and suggests that an osmotically sensitive receptor may have been affected by proteolysis.

Treatment of cells with RGDS peptide did not specifically reduce osmotic induction of luciferase activity which suggests that integrin-like binding motifs are not involved in osmotic-stress signal transduction pathway in tobacco cell suspension cultures.

The finding that a component of the osmotically-induced signal transduction pathway was sensitive to proteolytic digestion indicated that the osmotically-responsive moiety is at least partially external to the plasma membrane. Proteolytic digestion was also found to eliminate butyrate induction of luciferase activity (Figure 5.3.5) which suggests that this osmotically-sensitive moiety transduces the osmotic stress signal through the plasma membrane and that this moiety is

required for signal transduction. The suppression of osmotic induction of luciferase activity by pectin fragments as well as the suppression of osmotic- and butyrate-induction of luciferase activity by 1% BSA (discussed in Chapter 4) was a further indication that the signal transduction component was at least partially external to the plasma membrane and that the active site or active conformation required for signal transduction was made unavailable by interaction with either pectic fragments or BSA. The observation that pectic fragments did not suppress butyrate-induction of luciferase activity suggests that BSA may also stabilizes the plasma membrane and thereby affect the signal transduction mechanism.

These data support the hypothesis that an osmotic stress sensitive sensor may be present in the plasma membrane of tobacco suspension cells and is required for osmotic or butyrate induction of luciferase activity.

6 General Discussion

Bray (1993) classified genes induced in response to dehydration in three groups: (1) non-ABA-responsive genes - expression of these genes is induced by water deficit but not by application of exogenous ABA; (2) ABA-responsive genes: expression of these genes in ABA-deficient mutants is induced by water deficit or by application of ABA; (3) ABA-requiring genes: these genes are drought responsive, but are not expressed in ABA-deficient mutants. Although CDeT27-45 promoter is a drought- and ABA-responsive gene in *Craterostigma* (2), these responses are lost in CDeT27-45-*luxF* transformed tobacco. As CDeT27-45-*luxF* is not induced by ABA application or water deficit achieved by wilting, the CDeT27-45 promoter in tobacco does not belong in any of the above categories. Instead, the mechanism of CDeT27-45 induction in leaf tissue or in suspension cell culture was initiated by abrupt turgor decrease (Chapters 3, 4, 5).

In the Introduction, other potential signal transduction routes were discussed. A major difficulty in signal

transduction work is sorting out which observed phenomena are directly involved in a particular signal transduction from those that occur concomitantly, but effect general increases or decreases in physiological or metabolic rates in response to a given environmental situation. For example, ion channel regulation and stomatal opening occurs in response to ABA, pH and calcium fluxes but does not involve gene activation (Ward *et al* 1995).

The induction of CDeT27-45 promoter by abrupt decreases in cell turgor (Michel *et al* 1993, Chapter 3) provided a system in which to investigate an osmotically induced signal transduction mechanism.

6.1 Induction of CDeT27-45 promoter in *Nicotiana tabacum* transfected BY-2 protoplasts or transformed SR1 plants.

Chapters 2 and 3 describe the activity of a CDeT27-45-*luxF* in transfected BY-2 cells or in stably transformed tobacco. CDeT27-45 promoter activity was found to be weak in BY-2 protoplasts and increased only 2-fold by ABA. In transformed tobacco, the promoter was not induced by wilting or the application of exogenous ABA to leaf tissue but was only expressed in maturing seed. This is in contrast to the observed activity of this promoter in

Craterostigma plantagineum (Bartels *et al* 1990).

The activity generated by an auxin-driven promoter (Båga *et al* 1991) carried by the same transformation vector used with CDeT27-45, was two orders of magnitude stronger in both BY-2 cells and transgenic tobacco. This finding contrasts with the observed weak activity of CDet27-45 promoter in tobacco BY-2 protoplasts and complete lack of activity in transformed tobacco leaf cells. One possible explanation for this observation is that required trans-acting factors which regulate CDeT27-45 activity are either not present or are not activated in either BY-2 or tobacco leaf cells.

Another possibility is that the CDeT27-45 promoter must integrate into chromatin areas which become decondensed in direct response to specific stress signals. Therefore, an initial step in this model of the signal transduction pathway requires a change in chromatin structure from transcriptionally inactive to active. A recent study by Frisch *et al* (1995) suggested that chromatin structure change was involved in seed-specific transcriptional activation of bean β-phaseolin (*phas*) promoter expression in transgenic tobacco. They reported ectopic expression of the *phas* promoter in transfection assays of tobacco vegetative tissue and in seed from transformed tobacco,

6.2 A model of osmotic stress signal transduction in tobacco leaf cells.

Figure 6.1 The plant cell wall-plasma membrane continuum

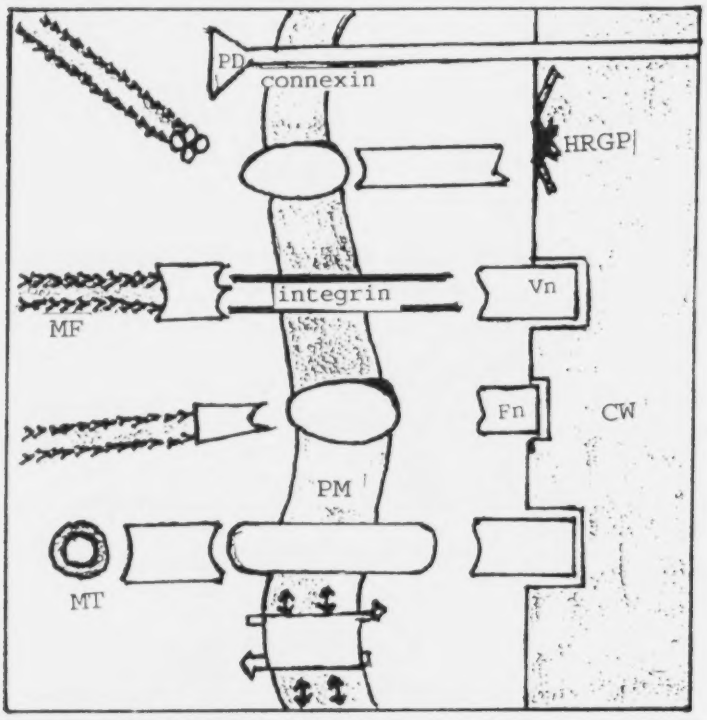


Diagram adapted from Roberts 1990, Wyatt and Carpita 1993

A schematic diagram of molecules that mediate connections between plant cell wall and cytoskeleton. Shaded elements are known: cell wall (CW), plasma membrane (PM), plasmodesmata (PD), hydroxyproline-rich glycoproteins (HRGP), actin microfilaments (MF), microtubules (MT), fibronectin (Fn), vitronectin (Vn). Some of the proteins which are antigenically homologous to those in animal systems are labelled.

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Based on other work, and my observations, I propose the following model for osmotically induced signal transduction.

The first event that occurs is a change in the fluid state of the plasma membrane. This can be in response to plasmolysis, the result of hyperosmotic stress, or as the result of 10 mM butyrate treatment. The observed decrease in membrane resistance due to hyperosmotic stress (Reid and Overall 1992) and the increased neutrophil membrane fluidization after treatment with propionic acid (Yuli and Oplatka 1987) support this suggestion. The presence of BSA may dampen the effective level of membrane fluidization by non-specifically binding to and stabilizing the plasma membrane.

Membrane fluidization may affect proteins involved in further signal transduction. Plasma membrane enzyme activities have been shown to be affected by membrane tension changes. For example, the activity of plasma

membrane phospholipase A has been shown to be increased by a decrease in membrane tension (Einspahr *et al* 1988) and phospholipase A products have been shown to activate NADH oxidase (Brightman *et al* 1991).

The osmotic stress signal is transduced via the actin cytoskeleton which connects the plasma membrane to the nucleus. For example, yeast actin cytoskeleton mutants are unable to respond to osmotic stress (Chowdhury *et al* 1992).

Data which supports elements of this model was presented in Chapters 4 and 5 and is summarized below.

6.3 CDeT27-45 was found to be induced in transformed tobacco leaves and in suspension cultures generated from transformed tobacco leaves, by osmotic stress or the application of short chain fatty acids.

Chapter 4 documented the induction of luciferase activity in response to osmotic stress as well as induction in the presence of short-chain fatty acids such as propionate and butyrate. The observation that luciferase activity was induced by butyrate concentrations greater than 1 mM suggests a general effect such as increased fluidization of the plasma membrane. Butyrate in 30FC medium with pH

adjusted to 7 resulted in a greater induction of luciferase activity than at pH 5. As the pH of tobacco cell cytoplasm has been reported to be 7.2 - 7.5 (Guern *et al* 1991), the phenomenon of proton-import by butyrate (i.e. acid loading) is unlikely to be the mechanism of signal transduction. As phospholipase A_2 activity has been shown to increase in response to decreased membrane tension (Einspahr *et al* 1988) and the products of phospholipase A_2 are fatty acids and lysolipids, osmotic induction may be mediated through the effects of released fatty acids/lysolipids on other proteins in the signal transduction pathway. The fact that BSA, which stabilizes membranes as well as proteins, eliminates osmotic-induction and decreases butyrate-induction of luciferase activity supports this model.

6.4 The cytoskeleton is involved in osmotic- and butyrate-induction of luciferase activity.

The finding that treatment of CDeT27-45-*luxF* transformed suspension cell cultures with cytoskeleton disrupting agents such as cytochalasin D and acrylamide, resulted in reduction in luciferase activity, supports the involvement of the cytoskeleton in osmotic stress signal transduction. While uniform agreement on the existence of intermediate filaments in plant cells is lacking, there

is much evidence of antigenically similar proteins. These proteins may be the ones which acrylamide treatment affects. The finding that treatment with either cytochalasin D or acrylamide post- osmotic or butyrate induction did not eliminate the response, indicates that the effect is not due to general toxicity.

6.5 Osmotic or butyrate induction of luciferase activity involves a protein receptor external to the plasma membrane.

Chapter 5 documented the finding that proteolytic digestion markedly reduced osmotic and butyrate induction of luciferase activity. Digestion with endopolygalacturonase (Y23 or PG) also reduced induction of luciferase activity in response to osmotic stress, but not in response to induction by butyrate. The products of Y23 or PG digestion did not induce luciferase activity but they did suppress induction. Conditioned medium in which tobacco suspension cells were first digested, then removed, contained heat stable moieties which suppressed osmotic but not butyrate induction of luciferase activity. This suggests that pectic cell wall fragments may be binding to a receptor, thus preventing signal transduction, but that the pectic wall fragments do not stabilize the plasma membrane. These findings support the

suggestion of a turgor-responsive receptor associated with the plasma membrane. The putative receptor is not only susceptible to proteolytic cleavage, but also to suppression through pectic fragment binding.

6.6 Future work on osmotic and butyrate induction of luciferase activity in tobacco suspension cells.

The next step in CDeT27-45 promoter activation in tobacco suspension culture cells is unknown. Are there turgor-responsive *trans*-acting factors which are activated in response to decreases in cytoskeletal tension and initiate transcription. Alternatively, is luciferase gene activation the result of induced chromatin decondensation and general transcription-protein complexes binding, such as the high mobility protein complex (HMC) or low mobility protein complex (LMC) described in the Introduction? These protein complexes have been implicated in maintaining mammalian chromosome sequences in a transcriptionally active state (Czarnecka *et al* 1992, Tjaden and Coruzzi 1994).

The cytoskeletal involvement in osmotic stress signal transduction pathway presents a system in which to look

at relevant chromosome state preceding and immediately following induction of luciferase activity. Sogo *et al* (1984) and Lucchini and Sogo (1995) have used psoralen-crosslinking to DNA to distinguish between active and therefore psoralen-accessible rRNA genes and inactive rRNA genes which are psoralen-inaccessible due to the presence of nucleosome proteins such as histones. DNA fragments which have been psoralen cross-linked can be distinguished by their slower mobility during agarose electrophoresis. Conconi *et al* (1992) have used psoralen cross-linking to investigate ribosomal chromatin structure in tomato plant leaves and dividing cell cultures. Their results indicate that the majority of ribosomal genes are folded in a nucleosomal structure.

6.7 Concluding remarks

The results of the experiments in this thesis suggest that:

1. A drought- and ABA-responsive promoter (CDeT27-45) isolated from *Craterostigma plantagineum* does not respond to either droughting or exogenous application of ABA when transformed into *Nicotiana tabacum*. In tobacco, CDeT27-45 is highly expressed in mature seeds and is induced by hyperosmotic stress in leaf tissue but cannot be induced by the exogenous application of ABA. Although CDeT27-45

promoter contains motifs which have some homology to ABA-regulated motifs (Introduction, section 1.14), the lack of ABA inducibility suggests that these motifs are not functional in tobacco leaf tissue.

2. A turgor-responsive receptor may be present in the plasma membrane of tobacco mesophyll cells. This putative receptor may be activated in response to externally applied hyperosmotic stress. Based on the observation that application of millimolar amounts of short-chain fatty acids propionate or butyrate, which are known to affect membrane fluidity, results in the induction of luciferase enzyme activity, I hypothesize that the changes in membrane tension activate other components of the signal transduction mechanism.

3. The turgor response is affected by proteolytic digestion and is suppressed by pectic fragments. These findings suggest that if there is a turgor-responsive receptor, it is external or partly external to the plasma membrane.

4. The mechanism of hyperosmotic stress signal transduction also involves the cytoskeleton and/or cytoskeleton binding components. The mechanism by which the cytoskeleton, either directly or indirectly, can

induce specific genes, remains to be investigated.

From this data I have developed a hypothetical model which focuses on the importance of the plasma membrane and cytoskeleton in osmotic stress signal transduction in tobacco.

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